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INFLUENCE OF INTRADERMAL BCG ON THE BIODISTRIBUTION OF RADIOGALLIUM IN MICE

University — Université

UNIVERSITY OF ALBERTA

Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

MASTER OF SCIENCE (RADIOPHARMACY)

Year this degree conferred — Année d'obtention de ce grade	Name of Supervisor — Nom du directeur de thèse
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THE INFLUENCE OF INTRADERMAL BCG ON THE BIODISTRIBUTION OF RADIOGALLIUM IN MICE

by

© STEPHEN DOUGLAS MALLET-PARET

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS OF THE DEGREE

OF MASTER OF SCIENCE

IN

RADIOPHARMACY

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

EDMONTON, ALBERTA

SPRING, 1981

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR . . . Stephen Douglas Mallet-Paret . . . .

TITLE OF THESIS . . . Influence of Intradermal BCG on the Biodistribution of Radiogallium in Mice . . . .

DEGREE FOR WHICH THESIS WAS PRESENTED . . . Master of Science . . . .

YEAR THIS DEGREE WAS GRANTED . . . . Spring 1981 . . . .

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TO MY PARENTS

ABSTRACT

BCG treatment has been shown to alter the biodistribution of ^{67}Ga-citrate in mice through the development of a systemic granulomatous reaction. At intervals of two days, and four weeks, after a BCG treatment regimen of an interdermal injection every second day for a total of 6 doses, 48 hour distribution studies showed increased uptake of the radiogallium in the liver, lungs, spleen, and kidneys. The effect was more pronounced four weeks after the BCG treatment regimens. Good correlation was noted between the altered biodistribution of radiogallium and the presence of histological abnormalities in the same tissues. Autoradiographic studies confirmed that radiogallium accumulations were largely associated with BCG induced lesions. Whole body counting did not indicate any statistically significant differences in the rates of elimination of radiogallium from BCG treated and control mice. *In vitro* uptake studies indicated that the BCG organisms, when viable, accumulated radiogallium. These findings would indicate the need for caution in interpreting diagnostic radiogallium scintiscans in patients receiving BCG immunotherapy.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. A. Shysh who has guided me throughout this project. During this period he has not only been a source of encouragement and guidance but also a friend.

I should also like to express my sincerest thanks to Mr. Chris Ediss, Dr. B.C. Lentle, Dr. F.I. Jackson, Dr. G.R. Johnson, Dr. U.K. Terner, Miss Cornelia Turner, and Dr. D.F. Biggs, whose assistance is appreciated.

The financial assistance in the form of a Medical Research Council of Canada Studentship and a Fellowship in the Canadian Foundation for the Advancement of Pharmacy is gratefully acknowledged.

The members of the Committee have always been extremely willing to assist me in any way and I would like to thank them at this time.

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I. INTRODUCTION

I. INTRODUCTION

Gallium-67-citrate scintiscanning has become a widely documented technique for the detection and staging of a wide group of neoplastic diseases as well as for the detection of various abscesses and inflammatory lesions. One of the major limitations of radiogallium as a tumour scanning agent is its nonspecificity of uptake. Many studies appear on the elucidation of the processes by which tumours and inflammatory lesions accumulate ^{67}Ga, but the exact mechanism is still unclear. Of great clinical importance is the understanding of factors which may influence and thus interfere with the diagnostic utility of the ^{67}Ga-citrate scintiscan. Immunotherapy with Bacillus Calmette-Guerin (BCG) is often used in the treatment of selected cancers including early or metastatic melanoma. Adverse effects, including skin reactions, BCG induced pneumonitis and granulomatous hepatitis have been reported following the administration of BCG. It has been observed that Ga-67 scintigraphs of patients with multisystem melanoma who had previously been treated with BCG revealed unusually high blood concentrations of the radiotracer, abnormal diffuse pulmonary localizations and increased uptake at sites not corresponding with clinical evidence of metastases.

In an effort to relate such abnormal biodistributions of ^{67}Ga in BCG treated patients to a reaction from the immunotherapy, this research project reports on the use of a BCG treated mouse animal model for studies of altered radiogallium uptake in certain tissues.

Following BCG treatment, the tissue distribution of i.v. injected gallium-67-citrate in mice was studied and changes in tissue

uptake were correlated to the presence of microscopic lesions visible histopathologically and autoradiographically.

Based on reports that radiogallium localization in inflammatory lesions may be due, in part, to uptake by micro-organisms at the infection site, and prompted by the histopathological observations of BCG accumulations within the BCG induced lesions in this study, a series of *in vitro* incubation experiments were undertaken to investigate whether the BCG bacillus itself may bind radiogallium.

II. SURVEY OF THE LITERATURE

A. GALLIUM

1. Introduction

Gallium-67-citrate is currently one of the most widely used tumour-seeking agents. In 1969, while searching for a bone scanning agent with a longer physical half-life than gallium-68, Hayes and Edwards discovered that carrier free gallium was taken up by certain soft tumours[2]. Since that time, gallium-67-citrate scintiscanning has become established as a supplementary diagnostic technique for a variety of neoplasms[3-16]. Scintiscanning with this radiopharmaceutical also provides a baseline for periodic reevaluation of tumour location and spread that is not obtainable by other non-invasive techniques[9]. The non-specificity of ^{67}Ga-citrate uptake at tumour and inflammatory sites and infections is a limitation to the diagnostic accuracy obtained with this radiopharmaceutical. Since the biodistribution of ^{67}Ga-citrate may be influenced by many factors, a good understanding of such parameters is important in interpretation of diagnostic scans.

2. Physical Characteristics of Ga-67

a) Production of Ga-67

Ga-67 may be produced by several reactions, including $^{67}Zn(p,2n)^{67}Ga$, $^{67}Zn(d,n)^{67}Ga$, $^{65}Cu(\gamma,2\mu)^{67}Ga$, $^{75}As(p,8n)^{67}Ga$ [17-19]. Gallium-67 is usually produced in a cyclotron by bombardment of enriched zinc-67-oxide with protons, or ^{66}Zn with deuterons[17,18].

It is essentially carrier free because of this mode of production followed by separation by a cation exchange procedure[18]. Gallium-67 may also be produced by a spallation reaction induced by a medium energy proton beam on a thin target of arsenic. Using an 800 Mev proton beam to an integrated intensity of 1 mA-hr, Ga-67 production with a chemical yield of 12.37 % and a yield of 76 Ci have been reported[19]. Arsenic has a cross section of 28.4 mb but the problem with this mode of production is the radiochemical separation involved. The radionuclidic impurities present with this method are ^{61}Cu, ^{64}Cu, ^{67}Cu, ^{66}Ga and ^{72}Ga.[19] Gallium-67-citrate must be used in the carrier free form for use as a radiodiagnostic scanning agent[17].

b) Chemistry of Ga-67-citrate

Gallium can form several chemical species at different pH values. Gallium citrate is stable throughout a pH range from about 2 to 7.5. If the pH is raised above 7, gallium citrate dissociation increases with the formation of increasing concentrations of gallium hydroxide and gallate ions[20]. Gallium exists in three valency states (+1, +2 and +3) and only the +3 oxidation state is stable in aqueous solutions. The following hydrolysis reactions have been shown to occur in solution[20].

1. $Ga^{+3} + OH^- \longrightarrow Ga(OH)^{+2}$ $K_1 = 2.5 \times 10^{11}$

2. $Ga(OH)^{+2} + OH^- \longrightarrow Ga(OH)_2^{+1}$ $K_2 = 6.2 \times 10^9$

Using a 1:2:4 pyridine, ethanol, water mixture and Whatman No. 1 chromatographic paper and dilute NaOH and HCl to adjust the pH,

these three components may be separated. At a pH of 9, most of the gallium citrate hydrolyzes to gallate (Rf 0.65) and $Ga(OH)_3$ (Rf 0.00) with only a smaller peak of ^{67}Ga-citrate appearing at Rf 0.9[20]. The molecular structure of donor-acceptor complexes of gallium halides with ammonia as studied by electron diffraction and mass spectrometry have just recently been solved[21].

c) Radionuclidic Properties of Ga-67

This radionuclide possesses many characteristics that are considered ideal for scanning purposes[2]. ^{67}Ga decays by electron capture, with no beta particle emission, with a physical half-life of 78 hours, to stable zinc[22]. The main photopeak energies and corresponding percent abundances are 93 KeV (37.8 %), 184.6 KeV (23.7%), 300.2 KeV (16 %), and 393.5 KeV (4.8 %). In the literature, there appears to be considerable variation in the photon emission probabilities per decay of ^{67}Ga. The most recent values are listed below[22,23].

Table 1

Photon Emission Probabilities Per Decay of Ga-67

Energy in KeV	Probability
8.6	0.495000
9.6	0.069200
91.3	0.031400
93.3	0.387000
184.6	0.209000
209.0	0.023700
300.2	0.167000
393.5	0.046000
494.2	0.000673
703.6	0.000125
794.5	0.000525
887.7	0.001488

Because of the absence of primary particle radiations, the radiation dosage to the tissues is relatively low[24]. In humans, the total body dose is about 0.26 rads/mCi with the absorbed dose to the various tissues being as follows:[1,24]

Table 2

Radiation Absorbed Dose to Selected Tissues
From Ga-67-Citrate

Tissue	rad/mCi
Bone	1.30
Bone marrow	0.58
Liver	0.46
Gonads	0.43
Lower large intestine	0.90
Upper large intestine	0.56
Small intestine	0.36

Although the physical half-life of Ga-67 is usually considered to be 78 hours, there is also some variation in the half-life estimations[18,22,25].

Table 3

Half-Lives of ^{67}Ga

Method	energy (KeV)	T 1/2 (days)
Ionization chamber	-	3.2595
Ge(Li) 1	184.6	3.2617
Ge(Li) 1	300.2	3.2615
Ge(Li) 2	184.6	3.2538
Ge(Li) 2	300.2	3.2593

The first half value thickness of lead is 0.04 mm and in water the first half value thickness is 5.0 cm. The specific gamma ray constant for ^{67}Ga is 1.6 R/mCi at 1 cm.

The 184 and 300 KeV photons are of moderate energy and of sufficient abundance to be suitable for imaging with either rectilinear scanners or gamma cameras[2,10]. The 93 KeV photo-peak has also been used[11]. Scatter of photons from the higher energy emissions falling around the 93 KeV peak decreases contrast and degrades the quality of the image when scanning in the integral mode. For this reason scanning is usually performed with multiple pulse height analyzers[11].

Twenty-five percent of disintegrations result in the emission of an 84 KeV conversion electron[26]. These low energy electrons permit autoradiographic studies with this radionuclide[26-30], including macroautoradiograms with frozen section on X-ray film as well as microautoradiographs yielding a resolution of 2.6 u in deparaffinized sections and 6.9 u using the Stumpf technique (dry autoradiograph)[28].

3. Pharmacology of Ga-67-citrate

Ga-67-citrate in a carrier free form is non-toxic in the usual administered dose. In mice the LD_{50} for i.v. ^{67}Ga-citrate was reported to be 115 mg/kg[31]. In the rat and the dog the LD_{50} values were 220 mg/kg and 182 mg/kg respectively[32]. Albuminuria and glycosuria, anorexia, nausea, vomiting and diarrhea or renal damage have occurred with large doses (> 70 mg/kg) of i.v. gallium

citrate in humans[32]. Although adverse reactions to scanning doses of Ga-67-citrate in humans are very rare, severe itching, erythema and rash have been observed in some patients[32]. However, gallium nitrate, in present use as a cancer chemotherapeutic agent, results in the formation of renal precipitates which occlude tubular lumina in rats[31]. Carrier free ^{67}Ga for diagnostic studies, however, requires administration of less than 10^{-7} mg/kg in man. Toxicity has not been observed with current preparations[33].

4. Distribution of Gallium-67-citrate

Gallium-67-citrate is usually administered by the intravenous route and occasionally by the intraperitoneal route as it is poorly absorbed after oral, subcutaneous or intramuscular injection. About 30% of the radiogallium, once in the blood, binds to plasma proteins including transferrin and haptoglobin. It is loosely bound to albumin and other serum proteins. The soluble remainder is diffused throughout the extracellular space and is excreted by the kidneys[34].

Normally, following i.v. administration, gallium concentrates most highly in the liver. Spleen, bone, secretory mucosa (very often the nasopharynx) and the large intestine show high activity. Two to three days after administration, the highest concentrations, in humans, are seen in the spleen (1%), kidney (2%), liver (5%) and bone, including marrow (24% of the administered dose of ^{67}Ga)[34]. Bowels, adrenal and lungs also have a high concentration. Muscle, skin, fat, blood and brain retain little of the activity[33]. Urinary excretion is the major route of elimination in man. The effective

half-life in man is two days. There is considerable variation in the fraction of the dose eliminated in the urine and it would seem from a preliminary study that like americium, plutonium and curium, gallium is bound to citrate in urine[35]. The distribution of radiogallium in humans is affected by several parameters, including age, sex, food intake, radiation exposure, and the presence or absence of carrier gallium[36]. Addition of carrier causes an increased uptake in the bone and more excretion in the urine[34]. Breast uptake is not uncommon and may be extensive during pregnancy or in postpartum women[11]. The biological half-life of Ga-67-citrate is increased in females possibly due to the distribution and quantity of adipose tissue as well as uptake in the breast[12].

In children the spleen, thymus, and epiphyseal plates show increased uptake in most instances[12,15]. Fasting affects the distribution in normal animals, by increasing the uptake of radiogallium in the liver, spleen and bone, as well as increasing the whole body retention[36]. It has been suggested that fasting be imposed in any gallium study where an alteration in food intake may occur[36]. In general, there is a decreased uptake in irradiated target organs of radiogallium following therapeutic doses of radiation and i.v. injection of Ga-67-citrate.

5. Ga-67-citrate Scintiscan Procedure

For whole body scanning, Ga-67-citrate is usually administered intravenously in a dose of 0.05 mCi/kg of body weight[9]. This amounts to about 3.5 mCi for the average patient although doses of up to 15 mCi

have been used. Interpretation of scans may be inconclusive if complicated by the presence of the nuclide in faecal material[32]. Although recent investigations suggest the concentration of gallium is higher in the walls of the intestine than in the intestinal contents, laxatives are employed to cleanse the bowels. Some suitable regimens are listed below.

Table 4

Alternate Bowel Preparations Prior to
Ga-67 Scintiscanning

1) 60 ml castor oil p.o., Fleet [R] enema prior evening[4].

2) 280 ml flavoured magnesium citrate two evenings prior and a low residue diet[3].

3) 2 bisacodyl tablets or magnesium citrate saline enema[5].

4) 30 ml milk of magnesia and 5 ml cascara daily for 3 days[7].

5) 2 Bisacodyl tablets p.o. evening before; 1 Bisacodyl suppository day of the scan, and a saline enema[6].

Scanning is usually delayed 24 to 72 hours after injection of gallium-67-citrate because of the initially high blood background[8,11].

Commonly, three pulse height analyzers are set to encompass the 93, 184 and 300 KeV peaks. With two analyzers the 93 and 184 peaks are used. If only a single channel analyzer is available a window is set to encompass both the 184 and 300 KeV peaks[11]. Information density over the liver of from 100 to 500 c/cm^2 is used with a 2:1 or 5:1 minification[1,11]. An anterior and posterior scan

is performed from the head to the knees taking about 30 minutes to two hours[11]. In inflammatory and infectious disorders it is not uncommon to use a computerized double-tracer subtraction technique employing, for instance, both Ga-67-citrate and Tc-99m-sulfur colloid. Each image can separately be set to 100% and varying amounts of Tc-99m are subtracted from the Ga-67-citrate image using a double tracer subtraction technique. The advantage of double tracer subtraction scanning is that it is highly successful in enhancing hot spot visibility and producing information as to the anatomic location of the lesion[33].

6. Utility of ^{67}Ga-citrate for Tumour Scanning

Gallium citrate Ga-67 is used most commonly to determine the extent and presence of malignancies, primarily, Hodgkin's disease, other lymphoma and bronchogenic carcinoma.

a) Hodgkin's Disease

Hodgkin's disease was the first malignancy to demonstrate uptake of radiogallium[2]. In one study, gallium scanning was able to determine the presence and extent of 70% of all clinically and histologically proven sites of involvement with a false positive rate of 5%. Although a significant correlation exists between scanning accuracy and tumour histology, gallium scanning has not replaced present methods established for clinical staging, as it is not sufficiently reliable to be used alone. Of all the types of Hodgkin's disease, the overall true positive rate was reported to

be highest for nodular sclerosing Hodgkin's disease[6]. The majority of false negatives involve sites below the diaphragm, particularly the spleen, paraaortic nodes and intestine[5]. However, gallium scanning is useful as a complementary procedure in the initial staging of Hodgkin's disease as it occasionally points out sites of involvement, for instance intraclavicular, pectoral and mediastinal lesions, that other diagnostic maneuvers have missed[6]. Gallium scanning is considered to be essential when lymphangiography is contraindicated, as in chronic obstructive pulmonary disease, or in the detection of clinically inaccessible sites[5,6]. The overall accuracies of lymphangiography and gallium-67-citrate scanning have been reported to be comparable in the detection of iliac and para-aortic lymph node involvement[38].

The value of Ga-67-citrate in the detection of Hodgkin's lymphoma above the diaphragm and at unsuspected sites of involvement, particularly in the bone is of established value. Inclusion of a gallium scan during the initial staging of Hodgkin's allows detection of extranodal disease which may change the patient's stage and alter therapy. A baseline is also provided to which future scans may be referred.[11] Despite a detection rate of 70 to 80% of involved sites, patients may easily be rescanned at suspicion of relapse rather than starting reevaluation with a series of complex invasive techniques according to another study[11]. Failure of chemotherapy or radiation therapy to convert a positive scan to negative is considered to be conclusive as to the presence of residual or recurrent disease[1].

b) Non-Hodgkin's Lymphoma

Ga-67 scanning is of practical value in the localization of non-Hodgkin's lymphoma. The sensitivity in the detection of histologically proven lesions is reported by Bekerman and co-workers[4], to be as high as 84% which is significantly better than In-111-bleomycin. As in Hodgkin's disease, the detection rate is dependent on the location and the histology of the lymphoma. With an overall true positive rate of 60%, as reported by Horn et al.[6] gallium scanning of patients with non-Hodgkin's lymphoma is not significantly less accurate than scanning of patients with Hodgkin's disease[6]. The true positive rate in patients with lymphocytic lymphoma was only 13% in the same study, however the true negative rate for the same group of patients was 100%. It is known that histiocytes concentrate gallium much better than lymphocytes or granulocytes[11].

The accuracy of gallium-67-citrate scintiscanning for the detection of non-Hodgkin's lymphoma below the diaphragm is low[7].

As in Hodgkin's disease, gallium scanning is employed as a supplementary staging tool[5], and as a noninvasive means of re-evaluation and detection of lymphomatous tumours in sites that are clinically inaccessible[5].

c) Bronchogenic Carcinoma

Ga-67-scintiscanning of the chest is often performed in cases of bronchogenic carcinoma to assess spread of a malignant neoplasm or whether a tumour has spread to hilar or mediastinal lymph nodes. There have been no explanations for bilateral hilar

uptake of ^{67}Ga after irradiation[39]. Both surgery and radiotherapy (3000 rads) are commonly used to treat bronchogenic carcinoma and follow up the disease. It has been suggested that scintiscanning with this radiopharmaceutical should be used with caution in the evaluation of a radiotherapeutic response to bronchogenic and esophageal carcinoma as subjective improvement on the scan often does not match with objective improvement[39,40].

Detection of primary lung cancer, particularly squamous cell carcinoma has been very successful. Eighty-one percent of histologically proven sites were positive[12]. False positives have occurred due to pneumonia and sarcoidosis. Of the primary lung carcinomas, squamous cell carcinoma and undifferentiated carcinoma showed significantly more ^{67}Ga uptake than tumour cells in adenocarcinoma[12,45].

In a large series of 272 patients for whom the cell type was available, there were no significant differences reported in the sensitivity of a gallium scan for squamous carcinoma, bronchoalveolar and adenocarcinoma, small cell carcinoma and large cell undifferentiated carcinoma[40]. Grain counts of autoradiographs showed macrophages, granulocytes, plasma cells, and lymphocytes to have less radioactivity than tumour cells[12].

d) Tumours of the Head and Neck

In a study performed by Higashi[13], involving 25 patients, gallium-67-citrate was found to accumulate densely in malignant melanoma, squamous cell carcinoma, anaplastic carcinoma and malignant lymphoma located in the head and neck. Localization in adenocarcinoma

and adenoid cystic carcinoma of the head and neck was poor. Reduced uptake was noted after irradiation (^{60}Co 1500-6600 rads) or chemotherapy if the therapy was effective. Good correlation was reported between patient improvement as indicated by the Ga-67 scintiscan and reflected by the clinical picture[13].

Maxillary sinus carcinoma has been differentiated from chronic maxillary sinusitis using ^{67}Ga scintigraphy[41]. The latter weakly takes up the nuclide while the former is strongly positive. Differential diagnosis has been difficult as the clinical findings are identical (facial pain, swollen cheeks, nasal discharge, etc.). These recent results conflict with previous findings that showed a 100% false negative rate in patients who had been preoperatively irradiated for squamous cell carcinoma of the head and neck[1]. The full significance of diminished tumour accumulation of ^{67}Ga following irradiation or chemotherapy is not yet clear.

Recent animal studies have shown high uptake of Ga-67-citrate in ocular melanoma[42]. Forty-eight hours after injection, the tumour-to-eye, tumour-to-choroid and tumour-to-blood ratios were high enough to perform scintigraphy. Human ocular melanoma scanning has been reported to be unsuccessful[42]. Scanning of patients with a variety of orbital diseases has been performed clinically with some success[75]. Differences between malignant tumours, benign tumours and inflammatory diseases have been claimed to be recognizable based on the kinetics of radiogallium appearance at the ocular disease process[43].

e) Liver Scanning

Both Ga-67-citrate and Tc-99m-colloid concentrate highly in the liver. Focal defects or "cold spots" on Tc-99m-colloid scans have been investigated using Ga-67-citrate[1]. A positive gallium scan over the defect may indicate hepatic neoplasm and eliminate most non-malignant diseases (eg. fibrosis, cysts, benign tumours and amyloidosis)[1,3,44]. Abscesses and inflammation of the liver will accumulate radiogallium while hepatocellular disease is manifest as decreased liver uptake and increased bone uptake of gallium-67-citrate.

f) Other

A variety of other tumours have been evaluated using gallium-67-citrate scintigraphy. Testicular tumours have been evaluated with a sensitivity of 93% and no false positives in 46 patients[1].

Gallium-67-scintiscanning proved to be unreliable in determining disease sites in most sarcomata but was quite accurate in assessing disease sites in patients with malignant schwanoma, Ewing's sarcoma and rhabdomyosarcoma[45].

Tumours of the digestive tract, thyroid gland, bone, breast, and brain, have been evaluated with gallium scanning with moderate to little success. Predominantly oxyphilic parathyroid adenomas can be visualized with Ga-67-citrate scintigraphy[47]. Gallium-67-citrate has also been used to follow up focal involvement in acute leukemia[1,26].

7. Uptake of Radiogallium in Inflammatory and Infectious Sites

Uptake of radiogallium by non-malignant disease was considered a severe limitation of this radiopharmaceutical for tumour scanning. Ga-67 is a useful agent for the identification of inflammatory processes. Some non-malignant, usually inflammatory diseases, in which gallium uptake has been noted are listed below[48-80].

Table 5

Non-Tumour Conditions Leading to
Abnormal Radiogallium Uptake

pneumonia	lupus erythematous
abscesses	pyelonephritis
silicosis	osteomyelitis
sarcoidosis	rheumatoid arthritis
Reiter's syndrome	paget's disease
fractures	cystitis
cholecystitis	appendicitis
pseudomembranous colitis	colitis
acute bacterial infection	surgical trauma
peritonitis	chronic bacterial infection
ureterosigmoidostomy	nephrolithiasis
pneumocystis carcinoma	bleomycin toxicity
thyroiditis	inflammatory bowel disease
tuberculosis (active)	psittacosis
post pneumonectomy empyema	dermatomyositis
immunosuppressives	fibrosis

a) Kidneys

Gallium concentration in the kidneys was shown to increase during acute pyelonephritis, nephrolithiasis, ureterosigmoidostomy and renal microabscesses[48,49,50,51]. ^{67}Ga localization only occurs during the acute stages of an infection in pyelonephritic animals[51].

^{67}Ga localization was not found to be useful in resolving lesions with gross histopathological and bacterial evidence of pyelonephritis, in renal infection without associated pathological changes or in renal lesions due to thermal injury[48]. Using Ga-67 tomographic radionuclide imaging with multiplanes enables clinicians to locate sites of focal infection[50]. Ga-67-citrate accumulates in the normal functioning transplanted kidney due to subclinical inflammation with accumulation of lymphocytes and monocytes. The activity can be seen there as much as two months after transplant and must not be confused with kidney infection[50].

b) Pulmonary Inflammations and Infections

Gallium-67-citrate is considered to be of great value in the diagnosis of pneumocystis carinii pneumonia because of the difficulty in performing lung X-rays of sufficient quality in these patients[1]. Increased lung uptake of the radionuclide is also associated with bleomycin toxicity[51]. Accumulation of ^{67}Ga-citrate in the lungs may indicate an inflammatory process. Gallium imaging can help select those patients with lung infiltrates who need angiography[52].

Diffuse inflammatory processes such as pulmonary sarcoidosis, also produce positive gallium scans[51]. In some patients, the symptoms will improve and the scan return to normal upon administration of steroids, however gallium scanning was reported to be less accurate than X-rays in the diagnosis of the sarcoid patients[51].

Gallium-67-citrate has demonstrated a propensity to accumulate in inflammatory, neoplastic lesions and all culture proven pulmonary

infections except some phases of tuberculosis[53,54]. Uptake in granulomata and chronic infections such as tuberculosis is of great interest.

Some researchers believe that Ga-67-citrate scanning is an important work-up for tubercular peritonitis[55]. In the case of tubercular peritonitis these same authors believe that the accumulation seen is due to the presence of the isotope in lysosome rich histiocytes within the tubercular granuloma[55]. An aspergilloma, or fungus ball containing septated branching hyphae as has occurred in the lungs of patients treated for atypical pulmonary tuberculosis caused by Mycobacterium Kansasii have been shown to accumulate gallium[56]. Gallium studies may be useful in extrapulmonary aspergillus infections as well[56]. Whole body ^{67}Ga scintimaging has led to the diagnosis of dermatomyositis, an inflammatory disease of unknown etiology[57], sometimes occurring in the lungs. The use of Ga-67-citrate scanning offers a reliable means of screening patients with and monitoring the response to therapy of extrapulmonary tuberculosis[58]. Ga-67-citrate scanning, in the context of its pulmonary uptake, may be a valuable method in the detection of late-onset postpneumonectomy empyema[59].

c) Gastrointestinal Disorders

A frequent problem in Crohn's disease is the distinction between exacerbated inflammation which can be treated with drugs and an abscess which must be treated surgically[60,61]. In the search for abdominal abscesses, using Ga-67-citrate scintiscanning, false

positives may result from patients with large (often palpable) masses of short evolution and secondarily infected lesions such as hematomas and pseudocysts[52]. A patient with pseudomembranous colitis may accumulate ^{67}Ga in the colon[63]. Radiogallium is widely recognized as a valuable tool in the detection and localization of abscesses, as gallium can accumulate in metabolically active tissue including infection[64]. Uptake occurs in acute cholecystitis but not chronic cholecystitis or the chronically diseased fibrotic gall bladder[1]. Gallium scintiscanning of the gall bladder is an important adjunctive study in the evaluation of cholecystitis[65]. Localization of subphrenic abscesses by gallium-67 scintigraphy can result in positive scans in 6 hours with no need for delayed scanning[66]. Perhaps the most important use of Ga-67-citrate, in the context of infection, is in postoperative patients where surgically induced abscesses are suspected. An iatrogenic false positive, in this instance, would be the rarely occurring disease, starch granulomatous disease, that is, a granulomatous condition that occurs postsurgically as the result of starch from surgeons' gloves[67]. Ga-67-citrate scanning has been used to diagnose retroperitoneal disorders and the disease included as a factor which may alter gallium scans. Ga-67-citrate scintiscanning of retroperitoneal disorders was reported to be significantly better than computerized tomography, sonography, radiography, aortography and barium studies of the gastrointestinal tract in the diagnosis of retroperitoneal paraprosthetic infections sometime after aortic reconstructive surgery[68].

d) Liver Abscesses

Ga-67-citrate has been shown to localize in liver abscesses[69]. Using a double isotope study, the Ga-67 may be seen to fit the colloid gap of Tc-99m-sulfur colloid, indicating a different metabolism of the isotope or selective fixation of ^{67}Ga in an abscess[69,70]. Focal sources of infection, in the liver, or septic lesions are commonly localized with a high true positive rate[70].

8. Mechanisms of Localization of Gallium-67-citrate

Radiogallium scanning has been performed for about 11 years, yet researchers are still investigating why the radionuclide is taken up by tumour and inflammatory cells[11,37,51].

It is believed that gallium citrate administration intravenously is bound almost immediately to transferrin[80] in a fashion similar to that of indium chloride. From there, tumour uptake somehow increases to a maximum between 48 and 72 hours[11].

In the presence of many divergent theories it appears that there is both an active and passive mechanism of uptake of gallium by the cells, with the active uptake being the greater one. ^{67}Ga bound to transferrin is taken up more quickly by tumour tissue than ^{67}Ga bound to citrate and it has further been suggested that ^{67}Ga enters the cell after gallium transferrin combines with a cell surface receptor[81]. After intravenous injection of Ga-67-citrate, two chemical forms can be found in the blood; that of Ga-67-hydroxides and Ga-67-transferrin in percentages of 33% and 67%

respectively. In rats, the tumour uptake is identical for both intravenous and intraperitoneal administration, yet if the transferrin binding capacity is saturated, tumour uptake decreases but the tumour-to-blood ratio increases[11]. The EMT-6 sarcoma-like tumour of BALB+C mice is one of the most avid tumours for radiogallium, both *in vivo* and *in vitro*, provided the amounts of citrate are kept low[82]. When mouse serum is prelabeled with Ga-67-citrate, then injected, the uptake in EMT-6 tumours is greater than when Ga-67-citrate is injected directly, presumably due to transferrin-mediated uptake[81]. Some authors believe that accumulation of Ga-67-citrate in certain regions is related to the presence of dividing cells, *in vivo* labelling of circulating leukocytes or the deposition of ^{67}Ga in areas of low pH[80]. Endocytosis of protein bound ^{67}Ga, transfer of ^{67}Ga from transferrin to lactoferrin, and diffusion into hyperpermeable tumour cells have been cited as reasons for entry into some cells[80,83,84]. Lactoferrin has been detected by immunofluorescence, in tumour tissues from patients with Hodgkin's disease and a patient with Burkitt's lymphoma, and the spleen also has slight amounts of lactoferrin[85]. All of these take up significant amounts of radiogallium[85].

In vitro labelling of blood leukocytes (both granulocytes and lymphocytes) can occur by incubating anticoagulated whole blood with Ga-67-citrate[86]. There is minimal uptake by red blood cells[86]. Blood leukocytes labelled *in vitro* with ^{67}Ga offer a satisfactory gamma-emitting radioactive tracer that can be used to develop a technique for scintigraphic detection and localization of abscesses[85].

Some *in vivo* and *in vitro* studies suggest that ^{67}Ga concentrates in macrophages that surround tumour cells and inflammatory lesions[1]. One study showed that polymorphonuclear leukocytes do not significantly accumulate ^{67}Ga unless the plasma membrane permeability is disrupted[87]. Analysis of abscess content shows that most of the gallium is in the noncellular fraction and as well, ^{67}Ga accumulates in inflammatory lesions of agranulocytic patients[87]. Some researchers believe that this represents leakage of gallium through hyperpermeable membranes[87]. The gallium is assumed to be protein bound[87]. Human granulocytes accumulate Ga-67-citrate when incubated under anoxic conditions and exclude the isotope when oxygenated, offering a partial explanation for uptake in abscesses and some tumour masses[7].

Normally, high physiological uptake in the liver, spleen and bone marrow has been related to certain reticulo-endothelial cells, including histiocytes[11].

Intracellularly, gallium binds to particles known as gallium binding granules (GBG)[1], that appear, by electron microscopy to be lysosomal in nature, and located in the cytoplasm[3]. A statistically significant correlation has been shown between the presence of lysosomal enzymes of hepatoma cells and uptake of radiogallium, indicating that once inside the cell, gallium associates with lysosomes[89]. It is well known that radiogallium is avid for intracellular components, particularly lysosome like organisms[88]. In the Morris 5123C rat hepatoma, gallium has been shown to associate with a glycoprotein of a molecular weight of 45,000 daltons[90].

Gallium-67-citrate uptake studies with homogenized tumour cells that have undergone gel filtration indicate that about 50% of the gallium present is associated with a glycoprotein with a molecular weight of 4.5×10^5 daltons[80]. Since lactoferrin has a molecular weight of 9.0×10^5 daltons some researchers express doubts that ^{67}Ga uptake in tumour is due entirely to lactoferrin involvement[80].

Using both rate-zonal and isopycnic-zonal centrifugation, it has been shown that both rat tumour and mouse lymphosarcoma lysosomes accumulate ^{67}Ga. Particles can be identified enzymatically by their acid phosphatase and N-acetyl-α-D-phosphatase and N-acetyl-α-D-glucosaminidase content. Autoradiography of selected gallium-binding granule fractions showed silver grains concentrated over electron dense single membrane organelles[30], in the cytoplasm. A high content of cytoplasmic mitochondria in oxyphilic cells may explain the uptake of gallium-67 in oxyphilic adenomas[47].

A second class of GBG has been found in hepatoma homogenates[91]. These smaller particles, or microvesicles bind the largest portion of gallium, whereas, in the liver, the GBG lysosomes are the major binding component. Preferential association of ^{67}Ga with microvesicles may be indicative of a basic difference between normal and malignant tissue[91].

^{68}Ga and ^{72}Ga have been investigated for use in diagnosis and treatment of bone malignancies[80]. High resolution autoradiography has shown intracellular localization in tumour tissue rather than in the extracellular spaces and supporting connective tissue[25].

The affinity of malignant lymphoma including Hodgkin's disease for the nuclide may be tentatively explained by the abundance of reticuloendothelial cells, including macrophages, that are rich in cytoplasmic lysosomes and lysosome-like organelles[10]. Similarly, uptake in inflammatory disease may be secondary to binding of gallium by lysosome rich granulocytes and macrophages.

Gallium-67 is administered in the citrate form to prevent colloid formation, but the level of citrate has little effect on the distribution[1]. In animal studies, ^{67}Ga-nitrilotriacetate, salicylate and ethylene diamine tetraacetic acid had either the same distribution patterns or tumour-to-tissue concentrations suggesting an initial ionic localization[92,93].

Scandium can compete for plasma protein binding sites with gallium. Using 0.5 mg of scandium/kg of body weight, causes a rapid decrease in uptake of ^{67}Ga in all tissues except bone, kidney and tumours, producing high early tumour to non-tumour ratios[92]. There are presently no agents being used clinically that can increase tumour uptake of ^{67}Ga or decrease non-tumour background[92]. Scandium and iron dextran have been investigated. Desferoxamine (DEF), a biologically derived iron chelating agent, has been shown to produce early high tumour-to-blood ratios when administered 3 hours after a Ga-67-citrate injection in mice. The moiety found in urine after the injection is a Ga-67-DEF complex[83,84,94]. Human transferrin, down to 2 ug/ml can greatly stimulate uptake of both ^{67}Ga and ^{59}Fe [84]. Pretreatment with saturating amounts of non-radioactive Fe^{+++} cancelled its ability to promote ^{59}Fe uptake but not ^{67}Ga uptake.

This suggests different aspects of interaction of transferrin with cells[84].

It has been suggested that lactoferrin may be the basic intracellular gallium binding material in normal tissues and inflammatory lesions[80,95]. This is supported by the fact that granulocytes have a high concentration of lactoferrin, and the protein is also found in bone marrow, spleen, colonic mucosa, breast milk and tissues and lacrimal secretions, nasal secretions and seminal fluid, which normally accumulate ^{67}Ga [80,95]. As well, during gestation and lactation, lysosomal enzymes in milk and breast tissue increase coinciding with increased gallium uptake[80].

In addition to uptake by lactoferrin in polymorphonuclear leukocytes, the localization of radiogallium at inflammatory and infectious sites has also been ascribed to binding by bacterial siderophores[95]. Non-specific and facilitated uptake of radiogallium by bacteria, notably staphylococcus aureus, has been offered as an explanation for uptake in some infectious processes[96]. Uptake by polymorphonuclear leukocytes and bacteria, which are the main components of inflammatory lesions, has been studied extensively[95,96]. Some microorganisms which show significant uptake of gallium-67 are S. aureus, E. coli, S. faecalis and Salmonella typhimurium[96]. There appears to be a linear relationship between the uptake of Ga-67-citrate by the organism S. aureus and the concentration of Ga-67-citrate[96]. About 21% of all enzymes that have been investigated are associated with a metal cofactor[93], but gallium has never been shown to have any trace element function[93].

9. New Agents and Prospects

There are a number of other compounds to which radiogallium may be attached as a ligand. The porphyrins are potential ligands and have a metabolism similar to that of transferrin[97]. Ga-67-transferrin and Fe-59-transferrin when incubated with human melanoma cells, are taken up avidly and show some potential for uptake by tumours in humans[76]. By using the iodinated species of each of these it has been shown that the rate of complexation to the cell is equal to or greater than the rate of uptake for the ^{59}Fe compound but not for the ^{67}Ga compound[76].

In vivo, using a canine transmissible venereal tumour (TVT) as a model, the uptake pattern of Ga-67-citrate is different from that of Ga-67-transferrin despite the fact that most authors believe ^{67}Ga administered as the citrate binds to transferrin immediately upon injection, so gallium transferrin may be of potential use for early scintigraphy as the rate of binding is higher[98]. $^{67}GaCl_3$ administered in the presence of 1-hydroxy-ethylidene-1,1-disodium phosphate (HEDSPA) disappears rapidly from blood circulation and accumulates rapidly in the skeleton[99]. The most suitable time for bone imaging is approximately 6 to 7 hours after the administration of the radiopharmaceutical but, as yet, this has only been reported in rat and mouse studies[99]. 2,3-dimercaptopropionyl glycine (DMPG) and 2-mercaptopropionyl-l-cysteine (MPC) have been labeled with ^{67}Ga and the distribution has been investigated in mice. These agents localize primarily in the liver and kidney respectively and may have some potential for use as scanning agents in humans[100].

Although gallium citrate is not the ideal scanning agent, it has found a definite role in the initial assessment and subsequent follow-ups of many malignancies. Understanding the nature of false positives has led to better accuracy and also to an application in the detection of inflammatory diseases. Therefore an understanding of the mechanisms of localization should lead to a further increase in the interpretation and possibly the clue to finding an agent which will increase the specificity of gallium for certain sites.

B. B.C.G.

1. Introduction

BCG (bacillus Calmette-Guerin) is a viable avirulent strain of bovine tubercle bacillus commonly used for vaccination of selected tuberculin negative individuals against tuberculosis[101]. It was isolated in 1913 by Calmette and Guerin and attenuated over 11 years by 231 subcultures. At present, BCG is being used to a limited extent, and being investigated for use in the immunoprevention and immunotherapy of various forms of cancer[102]. Immunoprevention or immunoprophylaxis is the process of lowering the incidence of neoplasia by vaccination with immunomodulators such as BCG[103]. Immunotherapy is the use of agents to augment the immune response against existing tumours, primarily by activation of the immune system[104].

There are many different experimental and clinically used strains of BCG, and these are outlined below where TMC denotes an experimental strain[105]:

a. Glaxo
b. Tice
c. Connaught
d. Rijks
e. Pasteur (Scarification)
f. Pasteur (Immuno F)
g. TMC 1011 (Pasteur)
h. TMC 1012 (Montreal)
i. TMC 1029 (Phipps)
j. TMC 1032 (Tice)

These 10 strains were compared in a study to measure ability to suppress tumour growth of a rat sarcoma by regional application. The Connaught strain was found superior to all the others when compared for tumour suppression based on any parameter; (dry weight, number of total units, number of viable units)[105]. γ-Ray sterilized vaccine was surprisingly as effective as the viable organisms[105].

There is no significant difference in the number of colony forming units (CFU) when BCG is sealed under vacuum or under nitrogen. Four years storage at 4°C reduces the colony forming units to 81% of the initial values. Four weeks at 37°C reduces the colony forming units to 61% of the initial values[106]. Freeze drying has resolved the stability problem of BCG vaccine even with the rather low survival percentage (about 50%) for the organism providing good thermostability and assuring economical storage and transport[105].

In vitro, BCG organisms may be characterized by their:

a) opacity of suspensions
b) oxygen uptake
c) germination rate
d) culturable particles/uG[107]
e) ATP content[108]

For vaccination purposes, BCG can be administered by subcutaneous injection, intracutaneous injection, multiple skin puncture and superficial linear scarification[101].

BCG in investigation as an adjunct in the therapeutic management of cancer, has been shown to have a negative, neutral and occasionally positive effect on tumour growth under various

circumstances[102]. There are a number of other related agents used to modulate the immune system. The agents include the following;

a) BCG-SE or soluble extract of BCG which has been tested in in vitro studies against acute lymphoblastic leukemia[109]. Mathe and coworkers were the first to report the successful treatment of this disease by immunization with tumour vaccine in 1969[110].

b) MER, MER-BCG or Methanol extraction residue of BCG is a so called "non-specific" immunomodulator capable of activating several immune functions. Its most important biological effects are:

i) Increasing the resistance against infection which reflects its antimicrobial activity.

ii) Antineoplastic activity which seems to correlate best with the macrophage content of the tumour.

iii) Increase of the tolerance to chemotherapy and radiotherapy.

iv) General immunostimulatory activity by way of:

1) stimulation of the macrophage system

2) stimulation of lymphoid cells and humoral immune responses

3) other

v) Occasional induction of immunological enhancement of tumor growth. This may be less likely with MER relative to whole BCG vaccination.

vi) Occasional induction of immunosuppression on prolonged administration or with high doses.

vii) Anticarcinogenic properties which may be related to its auto immunosuppressive activities[111].

MER has reached the stage of being used in clinical trials for a number of neoplasia[112]. MER is prepared by phenol killing and acetone washing of viable solid BCG. The portion that is then methanol insoluble is referred to as MER. Typically MER contains 40% proteins or peptides, 3% soluble lipids, 17% bound lipids, 8% elemental nitrogen and less than 2% mycolic acids. Glutamic acid, glycine and alanine are the major amino acids as shown by amino acid analysis of the protein fraction[113].

c) PPD or purified protein derivative of tuberculosis and DNCB or dinitrochlorobenzene have been used in the skin testing of a number of cancers treated with immunomodulators. PPD and DNCB are also used to treat some skin cancers.

d) Emulsified cell walls of BCG, another fraction of the organism, was found to be at least as effective as BCG itself in treating established dermal tumours and microscopic lymph node and visceral metastases of guinea pigs[114-117].

e) Protein bound polysaccharide kureha (PS-K) has an immunostimulant action similar to that of BCG and is isolated from basidio mycetes. Thus far it has been shown to cause rejection of male skin grafts by female mice and to have some antineoplastic activity[118,119].

f) Coenzyme Q_{10} administered by intramuscular injection can increase the bioenergetics of lymphocytes as measured by an increase in oligomycin sensitive ATPase activity[120]. Most of the alternatives to BCG were designed to avoid the toxicity associated with its infectious properties[115,116].

g) Corynebacterium parvum, a formalin killed microbe, has some use in the treatment of neoplasias and it was found that C. parvum was able to eradicate certain 7,12-dimethyl benzanthracene induced tumours in the rat when given intralesionally. A moderate dosage of Corynebacterium parvum was found to be effective in treating post-surgical metastasis of a 13762 rat mammary adenocarcinoma where all doses of BCG tested were less effective[121,122]. This microbe has also been used in man.

h) Killed BCG in 1.5% carboxymethyl cellulose, which cured guinea pigs with established hepatoma. The process of healing was accelerated when endotoxin from salmonella typhimurium was added to the BCG bacilli[123].

i) BCG mixed with Pseudomonas aeruginosa, which can kill tumours by the process of innocent bystander necrosis better than BCG alone[124]. On the subject of microbial organisms, BCG has a suppressive effect on the development of syphilitic lesions and growth of Treponema pallidum in tuberculin-positive rabbits. It does this by stimulating macrophages[125]. BCG cell walls can protect cotton rats against experimental echinococcus multilocularis infections[126].

j) A recent addition to the list of antitumour products derived from BCG is delipidated hot water extract from Mycobacterium bovis strain BCG. This is also called HSA standing for hot-water soluble adjuvant. It has no observable side effects in mice but has yet to be tried in humans[127].

k) A vaccine composed of BCG cell wall skeleton and P_3 (6,6-trehalose dimycolate) which was attached to mineral oil droplets was evaluated for activity and toxicity when injected into tumour nodules of humans. About 48% of injected nodules resolved while the side effects noted were pain (26%), fever (52%) and ulceration (61%)[128]. Micrococcus lysodeikticus and a series of related polysaccharides have a negative effect on the proliferation of 21210 leukaemia[129]. There is some evidence, although retrospective, that BCG affords protection against mononucleosis in humans, but this must be confirmed by Barr-Epstein virus serology[130]. In Burkitts Lymphoma after chemotherapy and BCG the induced remissions are much longer with a higher Barr-Epstein virus titre[131].

l) A Japanese substance referred to as wax D has some immuno-potentiating action as well. Thus far it has been shown to cause a strong increase in antibodies to sheep red blood cells in CF_1 mice. Wax D also works by a humoral and cell mediated immunity with methyl cholanthrene induced carcinogenesis in C_3H/He mice. Wax D is an extract of M. tuberculosis $H_{37}Ra$ [132].

m) Peritoneal exudate cells which have a cytostatic effect when mixed with BCG-CWS (cell wall skeleton) as measured by thymidine incorporation into rat fibrosarcoma cells[133].

n) A nonliving soluble BCG fraction, isolated in Bulgaria and referred to as F-70 has met with modest success in the treatment of lung cancer[134]. There is some evidence that more than one immunostimulant or adjuvant used in combination may act in an interacting fashion by having a positive stimulating effect on more

than one element of the immune system as is the case with BCG and LPS (lipopoly saccharide) in R595.

o) BCG and LPS in R595 used in conjunction with a formula defined diet in rats has been reported to be effective[135].

p) NED 137, a functional biopolymer has found limited use in humans for adjuvant therapy of post surgical clones[136].

Some of the factors that contribute to success of immunotherapy have been identified, yet this remains a very active area of research. Much of the research in this field has been conducted on animal models which do not necessarily correlate with human studies, where the data is often controversial. Extensive clinical trials, based on the immuno potentiating and macrophage activating properties of BCG are expected to continue[137]. Using the excellent review article of Bast et al.[103] as a basis, an attempt will be made to review recent findings relating to "BCG and Cancer".

2. Animal Investigations

a) Immunoprophylaxis

Infection of animals with viable BCG organisms or non-viable mycobacterial products can inhibit subsequent tumour growth. The immunoprophylaxis of tumour transplant is possible if a systemic BCG infection has taken place at least one week before injection of tumour cells[138,139]. Pretreatment with the vaccine may facilitate tumour growth in some lines of neoplasia by unknown mechanisms. Guinea-pigs treated immunoprophylactically with the methanol

extracted residue (MER) of BCG also protected 40% of the animals from neoplasia[140]. MER treated animals developed specific cell-mediated anti-tumour immunity more rapidly than did controls[141].

BCG may also prevent formation of clinically detectable metastases in some models[142], but tumour grafts in animals have commonly not responded to systemic BCG immunotherapy[103,138].

b) Immunotherapy

Tumour grafts in animals have commonly not responded to systemic BCG immunotherapy[103,138]. An exception to this is bladder carcinoma, melanoma, colon cancer and xenografts in athymic nude mice treated by local application of BCG. Athymic mouse xenografts may be useful for testing the response of human tumours to immunomodulators[139]. Stimulation of existing tumour growth has been observed in some instances following BCG injection, and in one case was attributed "to the nutrient medium in which BCG was suspended"[143]. Stimulation may also occur when BCG is administered intradermally at a site distant from the nodule. BCG administered intradermally by scarification spreads to draining lymph nodes and on to the spleen and can be followed quantitatively. Increasing the size of the innoculation or pretreatment with cortisone increases the number of viable organisms that can be recovered as well as the rate of spread to the spleen[144]. Previous vaccination reduces the rate of spread. Large doses of BCG induce an earlier but not an ultimately stronger immunity than does even a remarkably small dose[107].

Complete tumour suppression has been obtained when BCG was injected within 24 hours of challenge with tumour cells, when mixed with tumour cells before injection, or when injected intralesionally into small established tumours[103]. The following are considered important factors governing the failure or success of BCG immunotherapy trials in animals:

i) Tumour Size

Firstly, the success of BCG is inversely related to the size of the tumour. Effective treatment can be expected if the tumour load is less than 10^5 cells to 100 mg;[142] unless the growth rate or rate of metastasis adversely affects the number of amenable cells. The cure rate of guinea pigs with growing intradermal transplants of a syngeneic hepatoma at a time when lymph node metastases were detectable by palpation was not demonstratably greater in animals receiving surgery and BCG, when compared to surgery alone[139]. BCG was ineffective in tumours over 2 cm in diameter when given preoperatively to Fischer rats implanted with 13762 mammary adenocarcinoma[145]. An optimal time interval for administration of BCG before surgery significantly improved the survival time.

ii) Immunocompetence[146]

The development of an immune response to mycobacterial antigens is required. Inability to become positive to purified protein derivative of tuberculin (PPD) after treatment with BCG has been associated with non-regression of tumour transplants. Immunosuppressive agents can delay tuberculin sensitivity[103].

BCG, however, may reconstitute a depressed immune system as has been observed with cyclophosphamide induced granulocytopenia in the mouse.

Prolonged treatment with BCG initially improves immunocompetence, as measured by lymphocyte transformation by mitogens and skin reactivity to sensitizing agents, but in the case of malignant melanoma immunological parameters may rescind to pretreatment levels with certain overly long protocols[147].

iii) Number and Viability of Organisms Injected

Less than 5% of pellicle grown mycobacteria are viable. When dispersed in a liquid culture media greater viability is obtained. When equal numbers of viable organisms from each method of culturing were tested, the dispersed culture proved more efficacious in regressing guinea-pig tumours[103]. However, no significant differences could be determined in the mean survival time of the guinea pigs with metastatic hepatocarcinoma when treated with various strains of BCG by scarification (fresh Phipps, Phioas, Pasteur and Tice; lyophilized Pasteur, Tice and Connaught)[148]. There is some difference in the adjuvant effect of the BCG preparations as measured by a lymphocyte trapping assay. Pasteur is a stronger adjuvant than either Tice or Glaxo[149]. Viability determinations are somewhat dependent on the media the bacteria were grown on. In a study of Connaught, Glaxo and Tice strains were grown on Middlebrook 7H-11 medium or Dubos oleic agar viability counts increased as much as 30 fold with the latter enriched media. These findings show a need to standardize the growing media on which BCG is grown for proper application in tumour immunotherapy protocols[150].

The number of injected organisms is critical. The immunostimulatory effects of BCG reach a peak at a moderate dosage level, and decline or may become immunosuppressive at large dosage levels[154]. Facilitation of, or suppression of metastatic spread is dose dependent and dependent on the time of administration[103].

iv) Proximity of BCG and Tumour Cells

Intralesional injection of BCG promotes tumour regression in many instances where systemic or contralateral injection has no effect[103,104,151,152,153,142]. In mice, the oral route of administration is not effective for B16 melanoma or leukemia[139,155]. When given orally in the guinea pig, orally administered BCG organisms were recovered largely from Peyer's patches, a little from the mesenteric lymph node and a small amount from the liver and the spleen. The order is reversed when BCG is given subcutaneously[156]. There was, however, no evidence that intraumour injection of MER was more effective in prolonging the survival time of BALB/c female mice with adenocarcinomata than was application of MER at a distal site[157]. For indeterminant reasons, naturally arising tumours were less susceptible to BCG immunotherapy than were tumours of spontaneous origin but in the same study in rats local application of BCG seemed to give the best therapeutic response[158].

v) Immunogenicity of the Tumour[159]

BCG can enhance the growth of weakly antigenic neoplasms. Lines of tumour cells with a large amount of immunogenicity and a low potential for metastasis are more likely to respond to BCG[103].

Tumour cells may be treated in vitro with hydrolytic enzymes (e.g. Vibrio cholerae neuraminidase) or Mitomycin C to increase their immunogenicity[160-162].

Tumour producing capacity, as opposed to the immunogenicity of the tumour, is referred to as oncogenicity. Ehrlich ascites tumour cells loose oncogenicity or become attenuated when propagated in tissue cultures and subsequent treatment with gamma-irradiation abolishes the immunogenicity. Admixtures of the attenuated tumour cells with BCG seems to restore the immunoprophylactic effect[158].

c) Effect of BCG on Carcinogenesis

"BCG can delay the appearance of or reduce the incidence of tumours induced by chemicals, virus, or radiation"[103] and also inhibit the development of adenomas, sarcomas, carcinomas and leukemias in animals. This protective effect is temporary and dependent on the time of administration and the dosage[103,154]. Mixing the carcinogen with BCG more effectively prevents carcinogenesis. Tumour formation can also be delayed by cord factor (trehalose 6,6 dimycolate), and MER which do not cause PPD sensitivity to the same extent as BCG[103].

Studies have shown the methanol extraction residue of BCG is effective in delaying carcinogenesis from a number of compounds notably 7,12-dimethylbenz (a) anthracene-induced rat mammary carcinoma[163].

BCG's ability to prevent carcinogenesis is thought to be related to its ability to stimulate immune surveillance[103].

3. Human Investigation and the Use of BCG

a) Immunoprophylaxis

The discovery of the inhibitory effects of BCG on carcinogenesis in animals has prompted investigators to determine whether childhood vaccination has had any effect on the incidence of leukemia. Unfortunately, all the studies have been retrospective and some of the protocols lacked adequate controls[103]. There is little evidence that vaccination with BCG at an age greater than one year prevents cancer[103]. Davignon and Rosenthal suggest that BCG vaccination reduces death from acute childhood leukemia by 50 to 85%[103]. Long term prospective evaluations are needed.

b) Immunotherapy

BCG has been investigated for use in human cancer in two general ways[103]:

i) Intralesional injection into clinically apparent cutaneous tumours.

ii) Systemic administration for clinically inapparent tumour deposits following, or in conjunction with, cyto reduction (surgery, radiotherapy or chemotherapy)[137].

More specifically, a number of routes of administration have been used and will be described briefly[164]:

1) Scarification:

BCG vaccine is instilled into a grid 5 cm by 5 cm scratched on the surface of the skin and the area is kept dry for 24 hours. The area is then washed carefully with soap and water.

2) Intralesional:

BCG is injected into a superficial tumour nodule which is aspirated several days later yielding fluid with necrotic tumour cells and purulent materials. Vital signs must be monitored closely for 12 hours after injection. Tumour death at the site of intralesional injection occurs by the process of innocent bystander necrosis but regression of contralateral tumours occurs indicating a systemic mechanism of action.

3) Intracavitary instillation:

Dilute suspensions of 1 mg/ml of BCG are instilled into the pleural or abdominal cavity after the intracavitary fluid is removed. The patient is rotated from side to side to disperse the liquefied BCG over the lining of the cavity.

4) Intravesical instillation:

This mode of administration is used in carcinoma or sarcoma of the bladder. A dilute suspension of BCG is introduced by catheter and the patient then retains the solution for 3 hours before voiding.

5) Intrapulmonary:

BCG suspension is placed in a glass nebulizer and delivered as an aerosol into the lungs thus placing it as close as possible to

pulmonary lesions. The dosage is repeated once a week for a 3 month treatment regimen.

6) Intradermal:

BCG is administered in the deltoid surface of the arm to convert a tuberculin negative person to a tuberculin positive person.

7) Oral:

BCG is mixed with a fruit juice and the patient drinks the mixture. Dentures are removed to prevent entrapment of the bacilli in the mouth. This is the most commonly used and convenient mode of administration for pancreatic, hepatic and gastric tumours[164].

Efficacy of BCG has been reported in acute myelogenous leukemia, malignant melanoma, colon cancer, lymphoma, head and neck cancer and others[137].

4. Immunotherapy of Cutaneous Tumours

a) Melanoma

Intralesional injection of BCG has resulted in dramatic regression of multiple intradermal metastases of malignant melanoma[103]. This method of administration involves great risks of severe side effects and is not routinely employed[165]. In a study of eighteen patients previously treated with oral BCG, then by intradermal BCG in a perinodular fashion for malignant melanoma only in 9 cases was treatment unsuccessful. The patients on such an immunotherapy treatment had longer remissions and survived longer when compared to those

treated with chemotherapy alone[145]. Dystrophy and necrosis of the tumour occur after intralesional injection. By 15 days after intralesional injection the necrotic foci with accumulation of histiocytes and lymphoid cells has converted to granular tissue that replaces the tumour[166]. In a prospective comparison of intralesional and multipuncture BCG in recurrent intradermal melanoma, only the former resulted in complete regression of the tumours, and had a much higher success rate than the latter[167]. As in animals, the tumours must be small and the patients must be immunologically competent. A two year study has shown that the incidence of metastasis in patients treated with BCG was half that of the control patients[168]. In those patients on immunotherapy who relapsed, there was a six month delay before reoccurrence. Intralymphatic administration of BCG as a single dose of 0.2-80 mg has been assessed in patients with advanced malignant melanoma[169]. This mode of administration is relatively risk free, yet ineffective due to the spread of the disease[170].

Both MER and PPD have been tried in the treatment of malignant melanoma using intralesional immune therapy. Both agents may work by the mechanism of innocent bystander necrosis secondary to nonspecific inflammation rather than immunological mechanisms since uninjected tumours did not regress[171]. Malignant melanoma of the mucous membranes is a rare disease and is fatal. In one case report treatment with BCG, autologous tumour cells and cytabrine produced a positive delayed hypersensitivity to PPD. There was difficulty in interpreting the immunological findings according to

the clinical conditions and in the final stages of the disease the number of T-cells decreased [172].

5. Immunotherapy of Residual Cancer Following Cytoreduction

a) Acute Lymphocytic Leukemia

Immunotherapy has resolved experimental murine leukemia if the number of leukemic cells is less than 10^5 [103]. Acute leukemia involves about 10^{12} leukemic cells at diagnosis[103], so cytoreductive chemotherapy must be employed first. Residual acute lymphocytic leukemia may be treated with BCG provided the patient's immunocompetence has not been altered by the chemotherapy or by the disease itself[103]. For instance, palliative radiotherapy or cyclophosphamide has shown to render some patients unresponsive to BCG [173]. Vincristine or Adamantidene in combination with BCG gave worse results than BCG alone [174]. BCG used alone during remission of acute lymphocytic leukemia induced with intensive chemotherapy has met with some success[103]. Use of BCG with allogenic tumour cells or maintenance chemotherapy requires further investigation.

b) Acute Myelogenous Leukemia

Immunotherapy combined with intermittent chemotherapy (Rubidomycin, cytosine arabinoside, and 6-thioquanine) prolonged remission of acute myelogenous leukemia when compared to full maintenance chemotherapy alone[103]. An annual vaccination has

proven successful in prolonging the disease free period of stage IA and IIA malignant lymphoma that had been irradiated locally[103].

Squamous cell carcinoma of the head and neck may be treated by combined chemotherapy and immunotherapy in the form of methotrexate (MTX), Bacillus calmette-guerin (BCG), and Isoniazid (INH), with addition of cyclophosphamide (Cytoxan) in refractory cases, yet this may produce no better results than MTX alone[144].

Surgery and BCG immunotherapy have been combined to treat primary or recurrent melanoma with promising results. Malignant melanoma metastasis of the bladder in one patient was eradicated by transurethral interlesional BCG[175].

c) Lung Cancer

Radical surgery remains the only choice in advanced squamous cell lung cancer while systemic BCG therapy may be of some use in the initial stages of the disease. Again, there is the advantage of delayed hypersensitivity to recall antigens being a prognostic indicator[176].

Local administration of BCG after radiotherapy can overcome both radiation damaged and overloaded host defense mechanisms through stimulation to the extent that they become capable of eliminating those malignant stem cells which survive irradiation[177]. Intrapleural administration of BCG after surgical resection of lung tumours increased survival in patients with limited tumour burden but was not beneficial in patients with more advanced tumours[178,179]. No

effect on survival of patients with nonresectable lung cancer has been noted[180]. BCG immunotherapy was reported to increase the hope of survival for patients with resectable squamous cell carcinoma of the bronchi (stages I and II) to 66% over two years, whereas the survival rate of controls was only 38%[181].

d) Breast Cancer

BCG has been evaluated as a potential treatment modality for stage II and III breast cancer since 1974. It is administered by scarification, following regional radiation therapy in combination with 5-Fluorouracil, adriamycin and cyclophosphamide. This particular protocol of chemoimmunotherapy was reported to be effective in prolonging the disease free interval and survival status of breast cancer patients despite the menopausal status[182,183]. (For postmenopausal patients with breast cancer, adriamycin combinations remain an effective treatment in spite of potential cardiotoxicity[184].)

e) Ovarian Carcinoma

The chemotherapy of ovarian cancer typically involves use of combinations of drugs, yet only a few trials have used non-specific immunostimulants. BCG by scarification apparently enhances the antitumour activity of adriamycin-cyclophosphamide combinations and again skin testing is a useful prognostic indicator[185].

f) Colorectal Cancer

After resection of all known tumour deposits the prognosis for Duke B-2 or C-carcinoma of the bowel was reported to be very poor, with 2/3 of all patients treated surgically and followed through with adjuvant chemotherapy or immunotherapy dying of the cancer[185]. Adjuvant therapy, postoperatively, with methyl CCNU plus 5-FU in an attempt to prevent recurrence of disease showed no difference in the first 18 months. The BCG and chemotherapy protocol showed a slight advantage after that time but the results are preliminary and may not be real. The theoretical advantage of oral BCG in this instance is thought to be stimulation of lymphatic drainage from the G.I. tract[186].

g) Bronchial Cancer

In a Canadian study of carcinoma of the bronchus treated with BCG orally, it was found that immunotherapy had no favourable effect[187]. The dosage was considerable (80 mg), but when the BCG group was compared to the control there was no difference in the length of time after treatment when clinical metastasis were detectable[187].

h) Advanced Cancer

Treatment of advanced cancer with immunomodulators or chemotherapy is still in its relative infancy. In a double blind study of eighty-four patients with advanced cancer refractory to conventional

modes of therapy two dosage regimens of intradermal MER were compared to a saline placebo[188]. There was no apparent clinical advantage or immunological benefit over the placebo. This was, however, a good study as it emphasized the need of appropriate controls in studies of clinical immunostimulants in humans. The parameters measured in humans before and after treatment were hemoglobin, leukocyte count, differential blood, a serum chemistry profile and a chest X-ray[112]. The immunological parameters measured were recall skin tests with mumps, streptokinase, purified protein derivative of tuberculin (PPD), Candida dermatophytin and diluent; lymphocyte blastogenesis with three mitogens (Phytohemagluttinin concanavlin A and PWM); assays of peripheral T and B cells; serum immunoglobulins IgA, IgG and IgM; and dinitrochlorobenzene (DNCB) sensitization or challenge if the patient had previously been sensitized[112]. In advanced or metastatic breast cancer a trial of 5-FU, adriamycin and cyclophosphamide was attempted with BCG or with the nonspecific immune-reconstituting agent levamisole. Overall, levamisole was better tolerated than BCG and easier to administer. Since the results indicated chemotherapy to be superior to immunotherapy, the results suggested that levamisole alone may be of benefit and that the therapeutic ratio favours the use of levamisole instead of BCG in combination with the chemotherapeutic agents[188]. In metastatic adenocarcinoma of the kidney there was no prolongation with respect to historical controls (5-11 months survival after single organ metastasis), but these results may be considered tentative[190]. In advanced head and neck malignancy there was no difference in survival to a particular therapy

of high dose methotrexate whether or not BCG was added[190]. In advanced or metastatic breast cancer BCG plus 5-fluorouracil, adriamycin and cyclophosphamide had a slight advantage over chemotherapy alone (76 vs. 73% responders)[191]. Fortafur, an analogue of 5-fluorouracil has been used as a substitute for 5-FU but there was no particular advantage over the FAC-BCG regimen[192].

i) Prostate Carcinoma

Trials of BCG therapy have been conducted in prostate carcinoma where there has been extensive effort to immunostage, i.e. measure the patient's immunocompetence and correlate this to the clinical stage. Both humoral and cell mediated immunity is measured and the composite of these measures is referred to as the immuno stage. The prognostic parameters measured for humoral immunocompetence are total and individual levels of defined serum proteins as well as electrophoretic and immuno diffuse properties of immunoglobulins and complement. BCG immunotherapy of prostate cancer resulted in no mortalities and minimal morbidity[193].

6. Adverse Reactions and Side Effects of BCG Immunotherapy

Local reactions to BCG include itching and ulceration at the site of injection[101]. Even vaccinating doses, as opposed to immunizing doses may cause this.

Systemic BCG infection may result from intravenous administration particularly in immunodepressed patients, and for that reason

this mode of administration is contraindicated[104]. BCG infection responds to isoniazid, para-amino salicylic acid, and ethambutal. An edematous lesion with infiltration of mononuclear cells referred to as erythema multiforme is a rare complication of the BCG scarification technique[194]. A basal cell epithelioma, which is a benign lesion is a side effect of BCG vaccination. The occurrence of this latter lesion is no less frequent than the benign lesions that follow smallpox vaccination[195]. Findings are pertinent as the alveolar macrophages are activated by pulmonary washings where serum and serum immunoglobulins G transuded from blood into alveolar spaces[196].

Erythema nodosum, granulomatous hepatitis, hepatosplenomegaly, jaundice and pancytopenia have occurred separately in a small number of cases following the administration of BCG. Repeated intralesional injection of BCG has resulted in anaphylaxis and death[93,172]. BCG given by the intralymphatic route can produce variations in white cell count, erythrocyte sedimentation rate and immunoglobulins, fever, lymphanginitis and lymph node enlargement[184]. In animals, even killed BCG may cause a pulmonary inflammation with granulomas, and hepatosplenomegaly when the organism was injected i.v.[197]. BCG also changes cytochrome P-450 metabolism in the liver of rats. This finding might lead to reconsideration of dosages of drugs in cancer patients treated with combined chemoimmunotherapy[198]. The imidazole carboximides, DTIC and BIC commonly used in cancer patients for treatment of malignant melanoma are just two drugs which undergo demethylation by the cytochrome P-450 system[199]. BCG infected mice are hyperreactive to endotoxin and become very hypoglycemic when challenged. This reaction

has been confirmed in humans[200,201]. However BCG and Corynebacterium parvum in immunotherapeutic doses have no effect on hepatic drug hydroxylation of diphenylhydantoin in humans[202].

Intradermal MER may cause ulceration and cutaneous inflammation when given in high doses[112]. It has the advantage over BCG of being non-infectious[113].

Intralesional injection of BCG is more prone to cause side effects, particularly in PPD positive individuals. Death has occurred within 24 hours. Side effects are related to both dose and sensitivity to PPD and the patient must usually be hospitalized for observation[171].

a) Possible Processes

The means by which BCG indicates tumour regression and suppression are unknown[101]. The immune system itself is far from being completely understood[108].

There have been a number of review articles published on the pharmacology of the modulation of immunity by BCG and its capacity as an antitumour agent. In general, in the immunotherapy of cancer, BCG has met with some success in animals and limited success in man. The effects of BCG on the immune system are multifaceted and a clearer understanding will result in a more rational basis for its use[203]. The ability of melanoma patients to respond to BCG is correlated to their ability to become reactive to antigens such as tuberculin PPD, DNCB (dinitrochlorobenzene), etc.[103,140,204]. A low blastogenic response to lymphocyte cultures isolated from patients treated with

BCG to mitogens phytohemagglutinin (PHA) and concanavalin A indicates a rapidly progressing disease[204]. A chronic inflammatory response or a delayed cutaneous hypersensitivity at the site of intracutaneous, intralesional injection is associated with tumour suppression[103]. A chronic granulomatous response contributes to tumour cell degeneration. In various attempts to identify the subfractions of the BCG organism that are essential for antitumour activity, it has been found that the ability of cord factor (trehalose 6,6 dimycolate) or CSW-1 (partially deproteinized cell wall skeleton) to cause suppression of tumour transplants when administered with BCG is related to the ability of these components to produce an optimal granulomatous response[103,161].

Footpad injection of BCG causes a marked augmentation of lymph node response to T-dependent antigens but not T-independent antigens in normal thymus bearing heterogenous mice. Intravenous injection does not increase the response to either T-dependent (e.g. sheep red blood cells) or T-independent antigens (e.g. DNP-derivatized Ficoll) in both nude athymic and normal thymic mice. The augmentation seen with footpad injections appears to be mediated by T-helper cells[205]. Intravenous injection of fresh BCG results in a transient increase in lymphocyte mitosis associated with epithelial hyperplasia of the guinea-pig thymus in less than 10 days[206]. By the time lymphocyte masses appear in the spleen, about 15 days post injection, the changes in the medulla of the spleen are more obvious[206].

BCG can activate lymphocytes[206,207] and macrophages, in humans. Success of BCG adjuvant immunotherapy of ovarian carcinoma is thought

to be attributable to the activation of intraperitoneal macrophages[208]. In mice, activation of macrophages can be measured by carbon clearance and occurs whether BCG is given by footpad injection or intravenously[209]. An immune induced interferon is produced in mouse blood after stimulation with BCG or BCG-CWS if the mouse has previously been sensitized with either of these. This interferon is slightly different from viral induced interferon and is capable of activating macrophages cytotoxic to L1210 leukemia cells[210].

Alveolar macrophages obtained from BCG immune Syrian gold hamsters can exhibit cytotoxic activity against tumour cells when rechallenged intratracheally with BCG 5 days prior to assay. Non specific irritants capable of inducing inflammatory lung exudates do not result in tumour cell destruction[211]. Comparison of the cytotoxic activity of macrophages and lymphocytes isolated from the peritoneal cavity of BCG-sensitized mice as measured by the inhibition of DNA synthesis, revealed that the effector cells were amongst the macrophages. Spleen macrophages were devoid of cytotoxicity, and spleen lymphocytes possess the same significant cytotoxic activity in both BCG-sensitized and control mice[212]. Lymphocyte stimulation has some prognostic value in immunotherapy patients. The ability of BCG to stimulate a group of killer T cells in some patients may also relate to its immunotherapeutic effects[211], yet animal studies indicate T-cells probably play only a minor role[212]. A study of the generation of killer lymphocytes or T-cells <u>in vitro</u> against human antologous leukemia cells with leukemic blastocytes and soluble extract of BCG has been performed using acute lymphoblastic

leukemia patients under the age of 18 years[109]. When cultured with mitomycin treated blastocytes, remission lymphocytes became significantly lytic for autologous leukemic blasts. In some cases when the autologous blasts could not generate killer lymphocytes the combination of autologous blast and soluble extract of BCG (BCG-SE) induced more intensive cytotoxicity than before. This is related to the antigenicity of the tumour as mentioned previously[109]. Schistosoma mansoni infection is suppressable in both intact and B-cell deficient mice by pretreatment with BCG[213]. There is some evidence for a soluble factor secreted by BCG-T cells that stimulates the migration of other T-cells which have a role in tumour suppression[214]. BCG, therefore, is capable of producing both a humoral antibody response and a cell mediated immune response as evidenced by Tuberculin-induced transformation of foetal blood lymphocytes[215]. Production of helper T-cells specific for ovalbumin has been reported in sheep at 105 days[216]. Cytolytic activity is also associated with a natural killer (NK) cell found in peritoneal exudates harvested from BCG-innovulated C57BL/6 mice. These cells were also found in unimmunized mouse spleens and mesenteric lymph nodes. It is thought that BCG may activate resident NK cells, resulting in de novo production of NK cells or resulting in "homing" into the peritoneum of cells normally found in other lymphoid tissue[215]. When killed BCG is injected i.v. in an oil-in-saline emulsion a chronic granulomatous pulmonary inflammation and splenomegaly result in C57BL/6 mice[197]. The splenomegaly is associated with a marked reduction of phyto-hemagglutinin and lipopolysaccharide induced spleen cell proliferation. On the other hand, the response

to these mitogens is not reduced in BCG-injected CBA mice. The effect occurs at the cellular level and moreover is a property of adherent spleen cells and cannot be unblocked by anti-thy-1 serum[197]. Adjuvant disease, a polysystemic noninfectious inflammatory process, may be suppressed in rats by the administration of BCG at various dosage schedules, indicating that BCG may also be immunosuppressive in rats. BCG produced a decrease in the response to phytohemagluttinin as well as changes in lymphocyte subpopulations and recirculation patterns[217].

Tumour specific antibodies are elevated by active immunization with BCG and tumour cells, but not with tumour cells alone[103]. Elevated antibody titres favour an anti-tumour response[103,110,159]. There appears to be a cross-reactivity between BCG and malignant cells, suggesting that at least part of the mode of action may be due to their shared antigenic components[218]. Using crossed immunoelectrophoresis with an intermediate gel in a BCG/anti-BCG system the reaction against thirty distinct components of BCG was recorded[219].

Non-specific augmentation of the immune response, stimulation of the reticulo-endothelial system as BCG spreads from the site of innoculation, and an antigenic relationship between BCG and tumour lines have been suggested as the mechanism of BCG mediated tumour suppression and regression[103].

7. Summary and Prospects

BCG has proven effective in the immunotherapy of some types of human cancer provided the patient is immunologically competent and the disease is of limited extent[103]. Intravenous use of immunomodulators

requires considerably more investigation in animals before instituting routine clinical use in humans[197]. The question for ethics committees is one of the risk of applying anthropomorphic principles even when results between different strains of mice contrast.

There are a number of other diseases, such as some of the autoimmune diseases (rheumatoid arthritis, Hashimoto's thyroiditis, and Crohn's disease, as well as recurrent herpes simplex labialis) that have responded to immune modulation with BCG or levamisole, but the risk to benefit ratio remains quite high[219,220].

The use of BCG for lung or colorectal cancer, which accounts for a large percentage of the mortality due to cancer has undergone preliminary investigations[139,156,178,179,185,184]. Significant yet only preliminary results indicate that BCG vaccination has a beneficial effect on residual Hodgkin's lymphogranuloma[221]. Until the clinical situations where BCG can routinely be employed are identified, use in patients with cancer is limited to controlled trials[103,137,145,181].

III. MATERIALS AND METHODS

1. Animal Model

Young adult male mice of the ICR strain, weighing 20-25 g were maintained throughout the course of the experiments on standard laboratory diet (Purina [R] laboratory chow) and tap water ad libitum. Under sodium pentobarbital (i.p.) anesthesia, at a concentration of 0.1 mg/kg body weight, the inferior dorsal area of each mouse was shaved and rendered alopecic by application of a depilatory agent (Neet [R] cream). The mice were randomized into control and BCG test groups. Immediately prior to inoculation, the skin sites were cleansed with alcohol swabs.

Doses of 1 mg of lyophilized BCG vaccine (Connaught Laboratories Ltd., Willowdale) in a volume of 0.05 ml of sterile normal saline were injected intradermally into the prepared dorsal area of each BCG test mouse every second day for a total of 6 doses. Injections were made at the same time each day to avoid any variation due to circadian influence[222]. Each single dose consisted of approximately 1.3×10^7 colony-forming units[177]. Such a dosage regimen was reported to be effective in reducing the size of MC-42 tumours in C3H/HeJ mice[175]. Mice in the control groups received injections of sterile saline in like manner. At time intervals of 2 days and 4 weeks following the last BCG dosage the test and control mice were injected with ^{67}Ga-citrate (5 μCi in 0.1 ml) via a tail vein and tissue distribution studies were performed 48 hours later. Each organ or tissue was excised, rinsed in saline, blotted and weighed and then fixed in a 10% formalin solution, assayed for radioactivity in an automated

well counter (Searle Model 1185 gamma spectrometer) and following radioactive decay, each sample was forwarded to the Alberta Agriculture Veterinary Services Laboratories for histological examination. Live mice treated with BCG were also sent to these laboratories for examination by bacteriological methods.

2. Autoradiography and Histology

BCG pretreated and control mice were injected intravenously with 500 µCi of Ga-67-citrate, then sacrificed 48 hours later and various organs and tissues excised. Frozen tissue sections of 12-18 µm thickness were cut (IEC model CT microtome-cryostat) and placed onto microscope slides. Alternate sections were stained with Haematoxylin and Eosin (H-E) and some selected sections were stained with Ziehl-Neelsen stain. Under darkroom conditions Kodak NTB-3 tracking emulsion was placed in a thermostatically controlled water bath at 40-43°C and diluted 2:1 with distilled water. The microscope slides with unstained tissue sections were carefully dipped into the liquefied diluted NTB-3 emulsion, drained, placed in a light-tight box containing drying agent and exposed for 7 days at 2-4°C. The autoradiographs were developed in Kodak D-19 developer (1:1 dilution in distilled water) for 4-6 minutes, fixed in Kodak fixer (1:4 dilution) for 10-12 minutes, rinsed in tap water and dried.

3. Radionuclidic Impurities

Samples of gallium-67-citrate (C.E. Frosst) were decayed to an activity of 0.5 μCi over a period of one month and were measured on a WINS-GeLi detector in a fixed geometry for an elapsed time of 4 minutes and then again for 10 hours. The samples had been applied to filter papers in a volume of 0.5 ml and carefully coated in Saran R wrap. A standard, with 12 γ emitting isotopes, was measured in the same geometry on the same day. From this, a calibration curve of percent efficiency versus energy in KeV was prepared in order to locate, identify and quantitate any long lived radionuclidic impurities present in the decayed gallium-67 solution.

4. Biokinetics of Whole Body Elimination

In an effort to determine if the overall rates of elimination of ^{67}Ga-citrate between BCG and control ICR mice were the same, the following study was performed.

Eight control and 12 BCG pretreated mice were injected i.v. with about 2 μCi of Ga-67-citrate. The mice were maintained on a standard laboratory diet and the bedding changed regularly to avoid contamination of the pelt with feces or urine. Plastic containers with wire mesh were constructed to hold the mouse for counting in a small animal whole body counter as described by Lyster[223]. A background count was made prior to and after each animal count and the plastic container was washed or changed frequently to avoid contamination. The count rate of each animal minus background on day 0 was

considered to be 100% of the total administered activity. The decay corrected values on each day were expressed as a percentage of the activity on day 0. Measurements were made on days 0 through 4 and 7 through 11.

5. In Vitro Filter Uptake Studies

Prior experimentation had shown that BCG exhibited little or scanty growth when incubated in nutrient broth (Difco, 4 g/500 ml) whether incubated in a stationary fashion or in a mixing mode. Addition of surfactants did not alter the poor growth in nutrient broth. Since nutrient broth is a common media for bacterial growth, experiments were performed to determine if gallium-67 added to nutrient broth solutions would be taken up by Gelman Type G.A. metricel 0.45 μ filters, in order to determine:

1. The volume (V) of nutrient broth to be filtered.

2. The absolute activity (A) of Ga-67-citrate to be added to the 50 ml of nutrient broth.

3. The rinse volume of sterile saline (R) to ensure minimum filter activity as a percentage of total activity filtered. Each of the three factors (V, A and R) used at three levels representing 27 treatment combinations in a simple factorial design:

Experimental Design for Membrane Filter Study

Factor	Level 1	Level 2	Level 3
Volume (V)	0.5 ml	1.0 ml	2.0 ml
Activity (A)	0.25 μCi	2.0 μCi	4.0 μCi
Rinse (R)	1.6 ml	4.0 ml	10 ml

The entire experiment was repeated three times so that each treatment combination was performed in triplicate. A simple factorial design was used to analyze the results and find the minimum filter activity as a percentage of the total activity filtered without employing an arcsin percentage transformation.

6. In Vitro Uptake of Ga-67-Citrate by BCG

A live suspension of BCG was prepared by adding 15 mg of lyophilized BCG (Connaught Laboratories Ltd., Willowdale) to 50 ml of 7H9-Broth with OADC enrichment (Difco Laboratories, Montreal) and grown for 3 weeks at 37°C. An aliquot was withdrawn and diluted with Hank's solution without bivalent cations to 10^8 cells per ml using a Kirby-Bauer optical standard. A portion of the diluted BCG suspension was autoclaved at 120°C and 16 psi for 30 minutes for the study of uptake of Ga-67 by heat killed cells. The adjusted BCG suspensions were centrifuged at 2000 x g for 10 minutes and washed twice with cooled Hank's solution. The cells were resuspended in Hank's solution to 10^8/ml and Ga-67-citrate was added to a concentration of 2 uCi/ml and incubated at 37°C with gentle shaking. Polypropylene containers were used for all Ga-67 procedures since radiogallium is known to bind to glass surfaces but not plastic [232]. At time intervals of 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 3 hours and 4 hours after addition of radiogallium, 2 ml aliquot samples were removed and filtered with positive pressure through a Gelman Type GA Metricel cellulose triacetate membrane filter with a pore size

of 0.45 μm (Gelman Instruments Ltd., Montreal) followed by a 10 ml rinse with Hank's solution. The filter and filtrate were analyzed for levels of Ga-67 radioactivity and the results were expressed as a percentage of the total radioactivity in the aliquot sample which was retained on the filter.

IV. RESULTS AND DISCUSSION

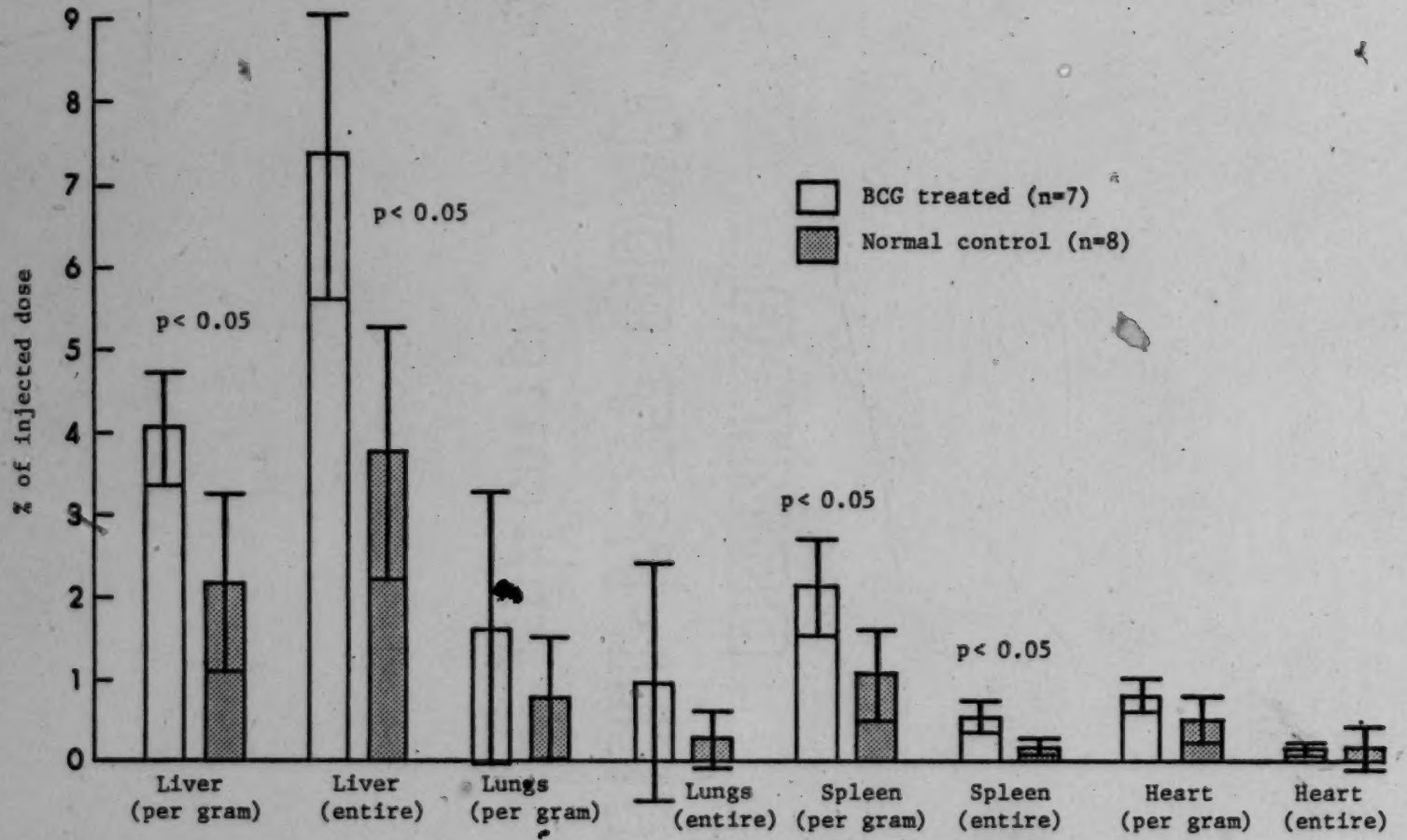
1. Animal Model and Histology

Preliminary experiments indicated that prior treatment with BCG resulted in an increased hepatic, pulmonary and splenic uptake of Ga-67-citrate in the inoculated mice. The magnitude of the uptake was varied widely and appeared to be influenced by several factors including the dose of BCG, the number of inoculations, and the time interval between the BCG treatment and the ^{67}Ga-citrate injection.

Figures 1 through 4 represent the results of a detailed 48 hour distribution study 2 days after the cessation of the 6 dose BCG regimen. Seven BCG treated and 8 control mice were used. The uptake of radiogallium was statistically greater, in the BCG group, in the case of the liver, spleen, kidneys, testes, stomach and g.i.t. and contents, and BCG injection site. Only in the case of the bone was the uptake of radiogallium greater in the control group. These preliminary results, 2 days after cessation of therapy, showed a need to increase sample size and perform further studies, with particular in depth study of the organs of interest: the liver, lungs, spleen and kidney.

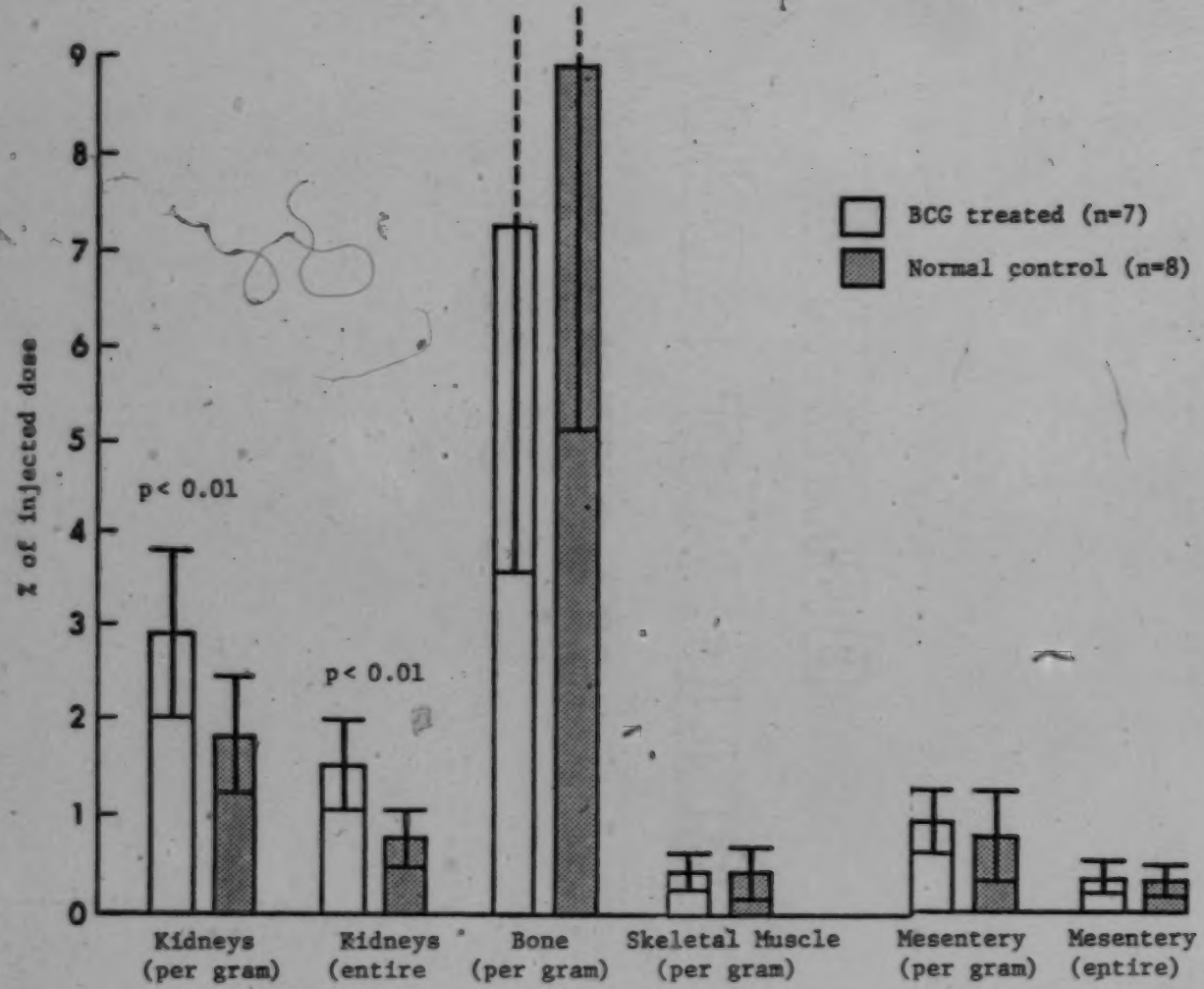
Table 6 and Figures 5 and 6 illustrate the 48 hour distribution of an intravenous dose of ^{67}Ga-citrate injected 2 days after the final BCG treatment in another study using larger numbers of control and BCG treated mice. An unpaired T-test was used to analyze the results. Statistically significant increases ($p < 0.01$) in the levels of radiogallium were seen in the liver, spleen and kidneys of the BCG treated mice. The uptake of ^{67}Ga in the lungs of the BCG test group was noteworthy because of the wide range of individual

Figure 1. Effects of BCG on the Biodistribution of ^{67}Ga-citrate in Mice



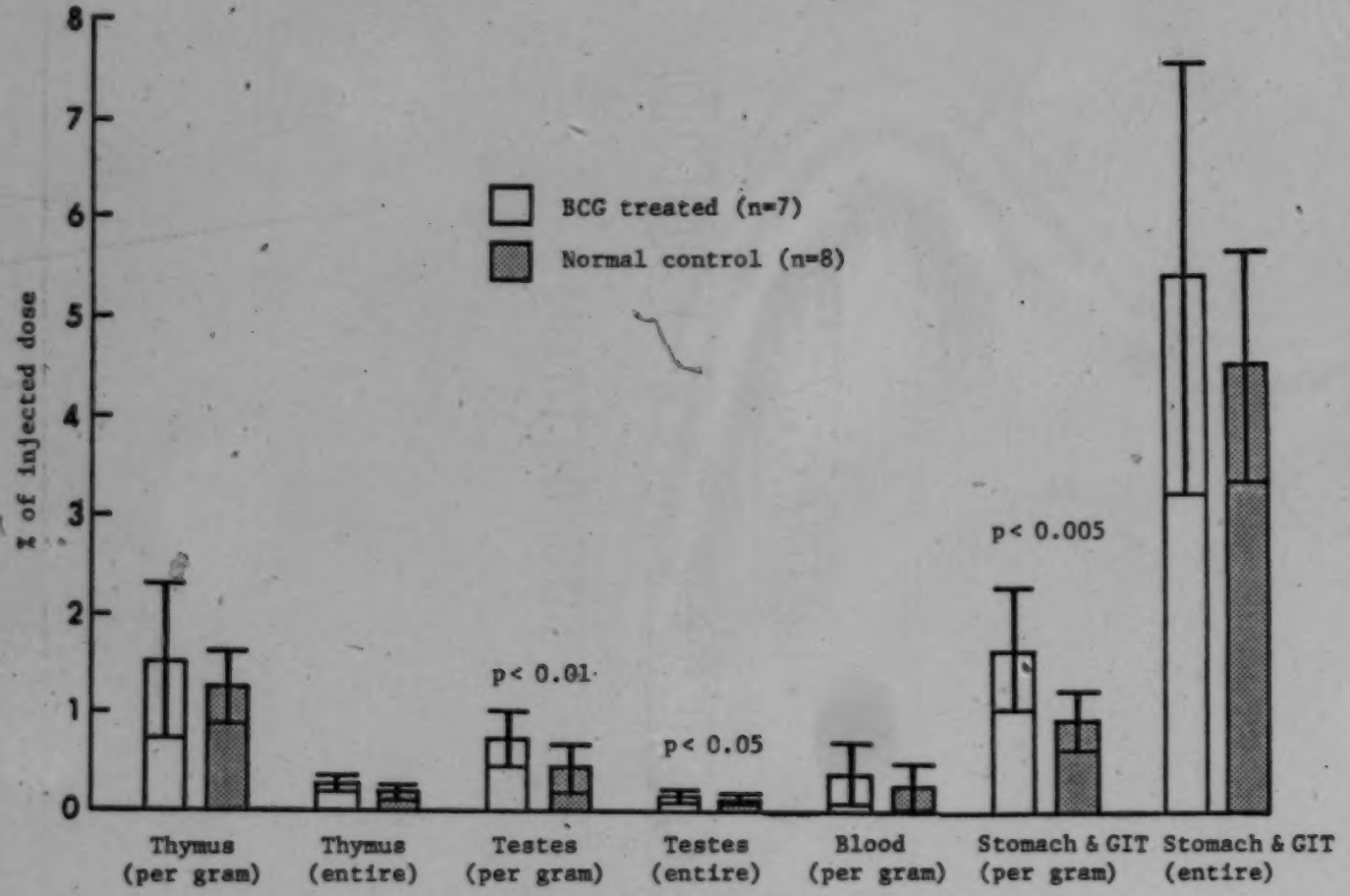
Each value represents the mean ± S.D. of normal and BCG treated mice 48 hours following i.v. injection of 5 μCi of Ga-67-citrate at a time interval of 2 days after the BCG treatment regimen.

Figure 2. Effects of BCG on the Biodistribution of ^{67}Ga-citrate in Mice



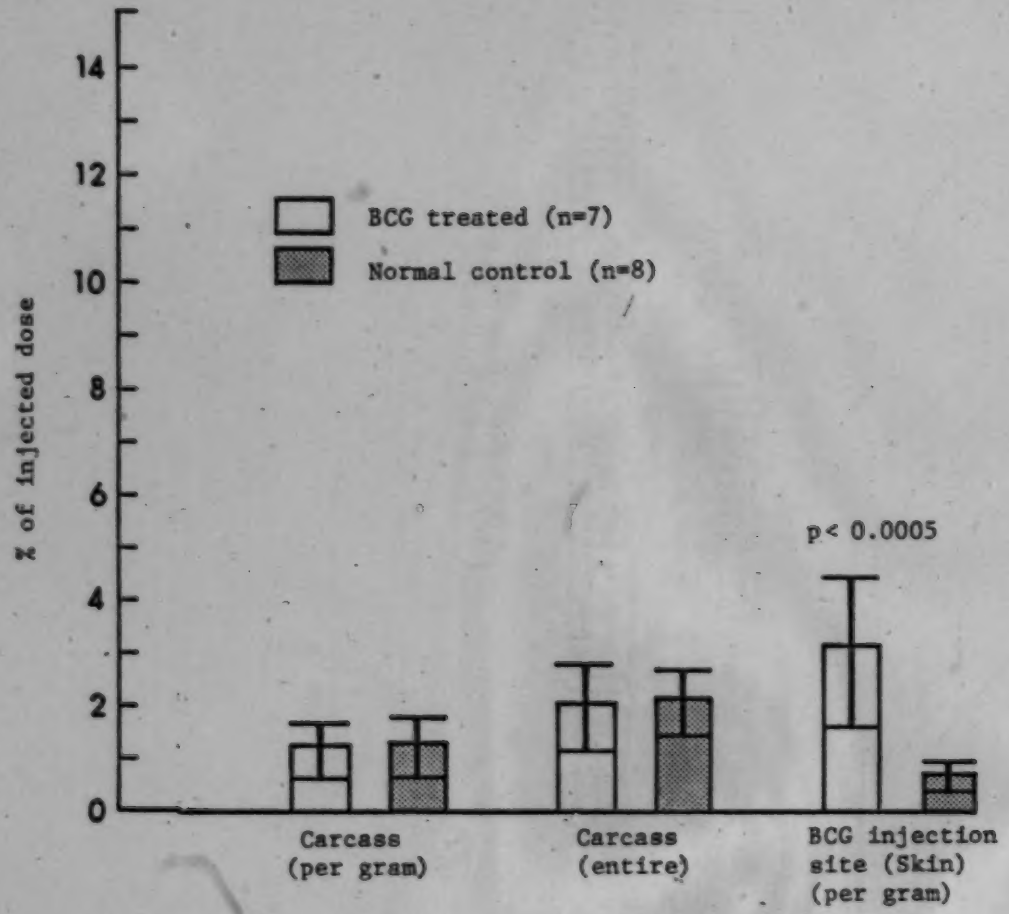
Each value represented the mean ± S.D. of normal and BCG treated mice 48 hours following i.v. injection of 5 μCi of Ga-67-citrate at a time interval of 2 days after the BCG treatment regimen.

Figure 3. Effects of BCG on the Biodistribution of ^{67}Ga-citrate in Mice



Each value represents the mean ± S.D. of normal and BCG treated mice 48 hours following i.v. injection of 5 μCi of Ga-67-citrate at a time interval of 2 days after the BCG treatment regimen.

Figure 4. Effects of BCG on the Biodistribution of ^{67}Ga-citrate in Mice



Each value represents the mean ± S.D. of normal and BCG treated mice 48 hours following i.v. injection of 5 µCi of Ga-67-citrate at a time interval of 2 days after the BCG treatment regimen.

Table 6. Tissue levels of ^{67}Ga in BCG treated mice [a]

	Percent of injected dose			
	BCG Treated [b]		Controls [c]	
Organ	Entire Organ	Per g of tissue	Entire Organ	Per g of tissue
Liver	5.83 ± 2.40***	2.75 ± 1.05*	3.15 ± 1.65	1.92 ± 1.50
Lungs	0.26 ± 0.17**	0.95 ± 0.62**	0.12 ± 0.06	0.47 ± 0.26
Spleen	0.40 ± 0.18***	1.34 ± 0.58*	0.14 ± 0.18	0.92 ± 0.73
Kidneys	1.21 ± 0.50***	1.91 ± 0.68*	0.71 ± 0.38	1.35 ± 0.83
Heart	0.08 ± 0.40	0.44 ± 0.21	0.05 ± 0.02	0.28 ± 0.16
Thymus	0.28 ± 0.13	1.58 ± 0.74*	0.23 ± 0.15	1.01 ± 0.75
Stomach and GIT	4.80 ± 2.60	0.77 ± 0.33	3.25 ± 1.78	0.54 ± 0.33
Skeletal muscle		0.27 ± 0.16		0.28 ± 0.12
Blood		0.38 ± 0.19**		0.21 ± 0.10
BCG injection site		1.90 ± 0.90***		0.65 ± 0.30

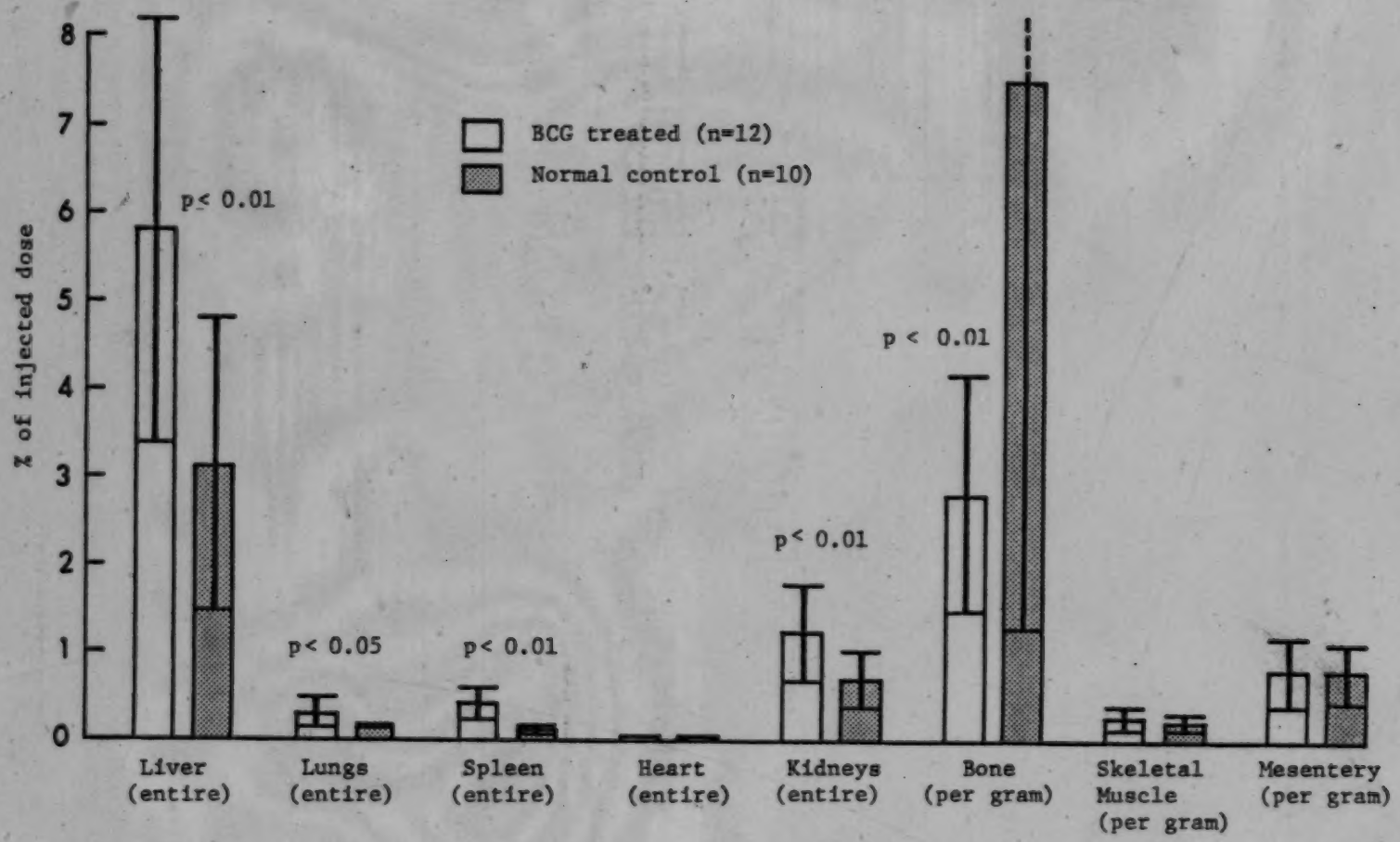
[a] 48 h following 5 µCi ^{67}Ga citrate i.v. administered 2 days after final BCG dose.

[b] Mean ± S.D. of 12 BCG treated mice.

[c] Mean ± S.D. of 10 controls.

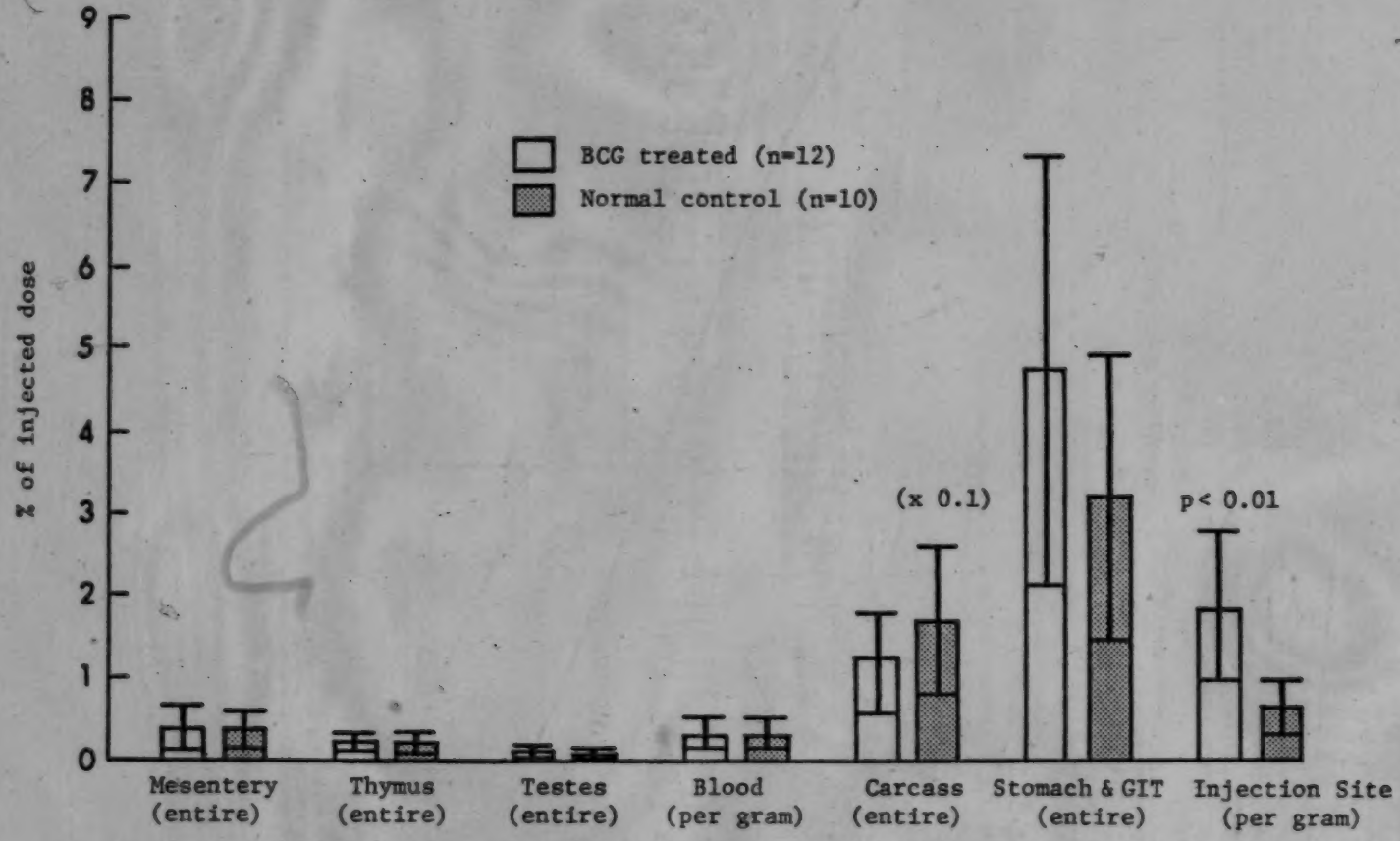
Statistically different from controls: *$p < 0.1$; **$p < 0.05$; ***$p < 0.01$.

Figure 5. Distribution of ^{67}Ga-citrate in Normal and BCG Treated Mice



Each value represents the mean ± S.D. of 10 normal and 12 BCG treated mice 48 hours following i.v. injection of 5 μCi Ga-67-citrate at a time interval of 2 days after the BCG treatment regimen.

Figure 6. Distribution of ^{67}Ga-citrate in Normal and BCG Treated Mice



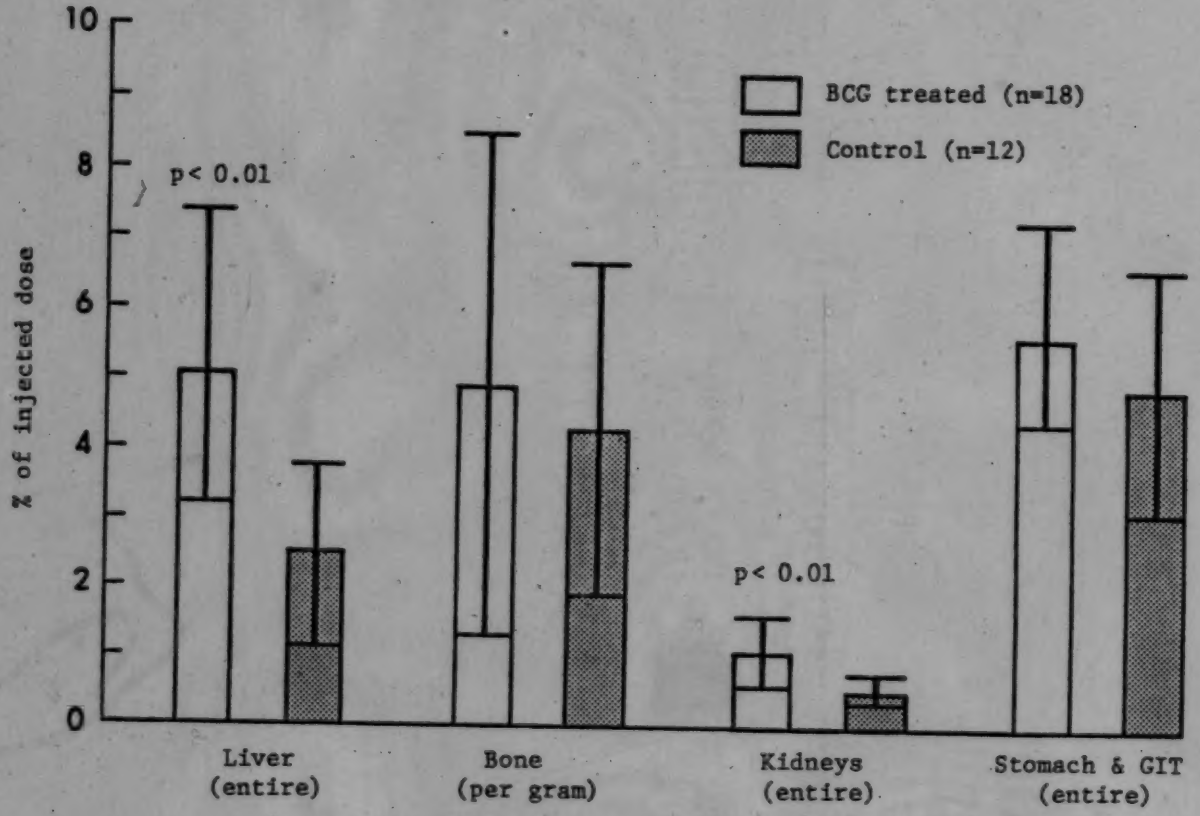
Each value represents the mean ± S.D. of 10 normal and 12 BCG treated mice 48 hours following i.v. injection of 5 μCi Ga-67-citrate at a time interval of 2 days after the BCG treatment regimen.

values observed in the 12 mice studied ranging from not different from, to a 6-fold increase over the mean control value. The effect of BCG treatment on the uptake of ^{67}Ga expressed on a per gram of tissue basis was somewhat influenced by concomitant changes in the mean weight of some of the organs. Thus, increases in radiogallium concentrations when calculated as percent of injected dose per gram of tissue were evident only at a $p < 0.1$ level in the liver, spleen and kidney. The mean liver weight in the BCG treated group was 46.3% higher than that of control mice, but the uptake of ^{67}Ga-citrate in the whole liver increased 85% above controls. Similarly, while the mean splenic weight in the test group increased 85.4% the uptake of the ^{67}Ga by the spleen was 185% higher than in the controls. BCG treatment resulted in an increase of 29.6% in the mean kidney weight and an elevation of 70.4% in the levels of radiogallium. Following BCG treatment the mean weight of the thymus decreased to 85% of the control group. Thus, while the radiogallium levels in the entire thymus gland did not change after the BCG regimen, the concentration on a per gram basis was higher. No significant changes in whole body weight, nor in weights of the lungs, heart or stomach and GIT were observed in the BCG group.

Organs from 3 of the BCG treated mice were examined bacteriologically. Under fluorescent microscopic examination for acid-fast bacilli, large numbers of bacilli were noted at the BCG injection site and lungs with moderate numbers in the liver, only a few in the spleen and no evidence of bacilli in the mesenteric lymph nodes.

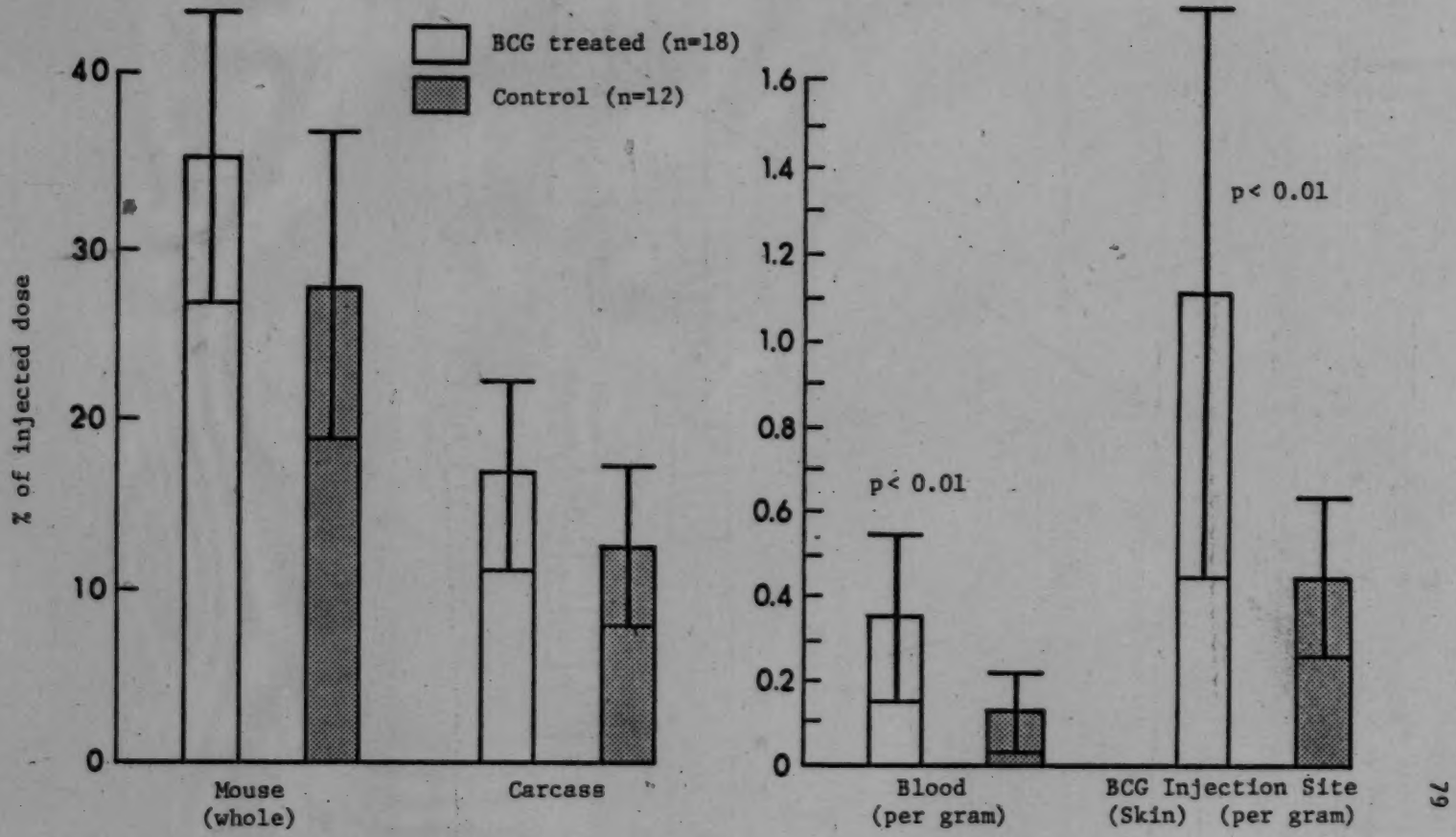
To allow the development of a full immune response in the interval between the last dosage of BCG and the ^{67}Ga distribution studies, another group of mice were treated with BCG on alternate days for 6 doses, left for 28 days and then injected with ^{67}Ga citrate. Figures 7, 8 and 9 and Table 7 summarize the data from this experiment. Tissue distribution studies conducted 4 weeks after the final BCG dosage indicated similar trends to those seen in the previous experiments except that increases in radiogallium levels were much more pronounced in the liver, lungs, spleen, kidney and blood. At this time, statistically significant ($p<0.05$) elevations in ^{67}Ga content of the heart were also observed in the test group. Because blood levels of radiogallium were consistently higher in the BCG treated mice, the possibility of increased tissue radioactivity due to contamination with blood was considered. However, since the blood was only about 6.5% of the total body weight, and since each of the tissues were rinsed with saline and blotted prior to radioactive counting, such increases in tissue radiogallium due to any residual blood were minimal. The histopathological findings, derived from the same samples which were analyzed for radioactivity, indicated that the presence of lesions was closely related to the levels of radioactivity in each organ. No abnormal lesions were found microscopically in any of the control animals. In the 18 BCG treated animals, 10 had an increase of radiogallium in the lungs by more than 2 standard deviations over controls. Of these, 8 had evidence of pulmonary granulomata and 2 of pulmonary edema. In the 7 mice with no increased uptake of ^{67}Ga in the lungs, no

Figure 7. Effects of BCG on the Biodistribution of ^{67}Ga-citrate in Mice



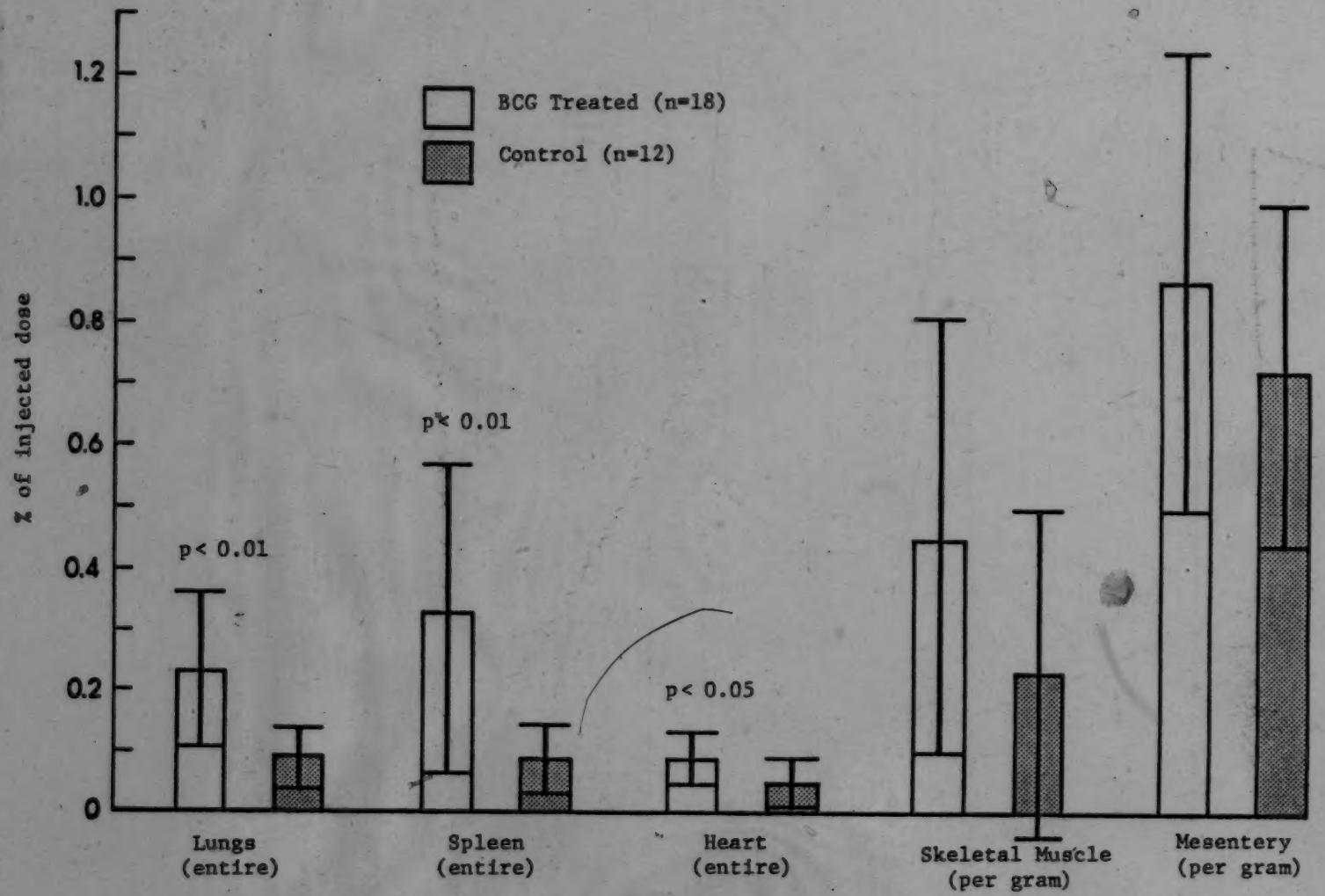
Each value represents mean ± S.D. of 12 normal and 18 BCG treated mice 48 hours following i.v. injection of 5 µCi Ga-67 citrate at a time interval of 28 days after the BCG treatment regimen.

Figure 8. Effects of BCG on the Biodistribution of ^{67}Ga-citrate in Mice



Each value represents mean ± S.D. of 12 normal and 18 BCG treated mice 48 hours following i.v. injection of 5 μCi Ga-67-citrate at a time interval of 28 days after the BCG treatment regimen.

Figure 9. Effects of BCG on the Biodistribution of ^{67}Ga-citrate in Mice



Each value represents mean ± S.D. of 12 normal and 18 BCG treated mice 48 hours following i.v. injection of 5 µCi Ga-67-citrate at a time interval of 28 days after the BCG treatment regimen.

Table 7. Effects of BCG Treatment on Biodistribution of ^{67}Ga Citrate in Mice [a]

Organ	Percent of Controls [b]
Liver	200.8 ± 85.7**
Lungs	243.5 ± 51.6**
Spleen	343.5 ± 55.8**
Kidneys	211.5 ± 69.5**
Heart	171.7 ± 101.8*
Stomach and GIT	114.4 ± 56.7
Skeletal muscle (per g)	122.8 ± 92.7
Blood (per g)	267.7 ± 90.9**
Injection site (per g)	255.4 ± 64.5**
Bone (per g)	113.3 ± 79.3

[a] 18 mice treated on alternate days for 6 doses; injected i.v. with 5 μCi of ^{67}Ga citrate 4 weeks later; tissue distribution at 48 h.

[b] Expressed as percent ± S.D. of [mean of BCG group (n = 18)]/[mean of control group (n = 12)].

* Statistically different from controls ($p < 0.05$).

** Statistically different from controls ($p < 0.01$).

microscopic lesions were seen, and in 1 animal, an increase in pulmonary levels of radiogallium was noted although no abnormal tissue changes were reported.

Correlation of histological findings in the liver with radiogallium concentrations showed that 12 of the 18 BCG treated animals had significantly increased uptake of ^{67}Ga; of these, 6 had evidence of granulomata, 3 showed foci of mononuclear cells, while in 3 animals, no abnormality was obvious. In the remaining 6 mice, there was no increased ^{67}Ga uptake by the liver, nor were any histological abnormalities seen.

Microscopic abnormalities of the spleen were present in all 18 of the BCG treated mice with 15 of those showing a significantly increased uptake of ^{67}Ga. In the histological study there were 12 cases of nodular necrotic areas, with pronounced changes in follicular size and shape and 6 with follicular hyperplasia. Three of the 18 animals showed no increased uptake of radiogallium in the spleen although in these 3, some changes in follicular size and shape were seen microscopically.

The relationship between increased ^{67}Ga uptake was less consistent in the kidney. In 12 of the 18 BCG treated mice, the radiogallium levels were much higher than in control mice; however, focal accumulation of mononuclear cells in the renal cortex was seen in only 3 of the cases while no histological changes were seen in the kidneys from the remaining 9 test mice. In 6 animals, neither elevation in radiogallium concentration nor any microscopic changes were observed.

The site of intradermal injection of the BCG was characterized by subcutaneous edema, moderate-to-severe granulomatous reactions or small abscesses in 13 out of 18 treated mice, 9 of which had significantly high levels of ^{67}Ga. In 5 animals, no significant lesions were observed at the BCG injection site and in 4 of these no increase in ^{67}Ga deposition was noted.

A number of procedures appear in the literature for the establishment of BCG immunized animal models with variations in doses, routes and sites of live BCG, BCG extracts and other related adjuvants. Although the intravenous injection of BCG has been effective in immunization of laboratory animals[225,226,227], the intradermal route of BCG was chosen in this mouse animal model in order to simulate this mode of initial BCG treatment in patients[228].

Successful immunotherapy by intradermal BCG injection has been achieved in mice, whereas, the gastric route was not effective[229]. The histopathological findings in the mouse model following intradermal BCG immunotherapy were very similar to those reported following intravenous BCG[226].

The altered biodistribution of radiogallium in BCG pre-treated mice appears to be relevant to the interpretation of unusual ^{67}Ga-citrate scintimages in patients receiving BCG immune stimulation. A very close correlation existed between the increases in concentration of radiogallium and histological evidence of BCG induced lesions in the same tissues.

The accumulation of ^{67}Ga at the site of intradermal injection of BCG was not unexpected since cutaneous reactions in humans[211,212]

and in animals[57] are well recognized and the affinity of ^{67}Ga-citrate for sites of inflammation and abscesses is well established.

Hepatic granulomata and granulomatous hepatitis have been reported in patients receiving BCG immunotherapy[159, 211] although acid-fast bacilli were rarely found in hepatic granuloma[245].

Significantly increased accumulation of ^{67}Ga concomitant with histopathologically visible lesions were evident in both groups of mice, those in whom tissue distribution studies were performed 2 days after the last dosage of BCG as well as 4 weeks later, although the increases in ^{67}Ga were much more pronounced in the latter group. Acid-fast bacilli in the liver of the BCG treated mice could readily be identified. Since the liver normally accumulates the radionuclide, increased liver uptake may go unnoticed in routine ^{67}Ga scintimaging.

BCG, after intradermal injection in guinea pigs, has been shown to migrate by lymphatic channels to the spleen at a rate dependent on the size of the innoculation[144]. Increased radiogallium uptake at this site may thus reflect an inflammatory response. Splenic histopathologic changes were noted in all the BCG dosed mice examined 4 weeks after cessation of BCG treatment. The numerous nodular necrotic foci and changes in follicular size and shape so commonly seen in the spleen and lymph nodes would account for the higher accumulation of ^{67}Ga at these inflammatory sites. The observation that BCG administration in mice[231] produces a sharp increase in the total number of spleen cells up to 16 days following immunotherapy may further explain radiogallium concentrations due to increased cellularity of the spleen. Follicular granulomata in the

spleen of the BCG treated patients were reported at necropsy [232]. Subjective assessment of increased splenic radiogallium uptake during ^{67}Ga scintiscanning may be hampered by the normally high accumulation of the radionuclide in this organ.

Although renal BCG-osis in patients on BCG immunotherapy has not been reported, the animal data presented here suggests this possibility in spite of the rather poor correlation between increased radiogallium levels and microscopic lesions.

Perhaps the most relevant finding is the increased radiogallium uptake in the lungs associated with BCG induced granulomatous pneumonitis. The fact that a high ^{67}Ga uptake and microscopic lesions were seen in only a few mice two days after the BCG dosage regimen would indicate that the pulmonary reaction may be an occasional phenomenon. However, data from the mice tested 4 weeks after cessation of the immunotherapy was much more conclusive.

Metastatic melanoma commonly involves the lungs where the presence of the disease may be detected by ^{67}Ga citrate scintigraphy. The possibility exists that increased pulmonary uptake of ^{67}Ga in melanoma patients on BCG immunotherapy may be due to the presence of melanoma or the inflammatory reaction to the BCG [231]. A study of 10 melanoma patients treated with BCG indicated that a diffuse increase in pulmonary radiogallium uptake and a low single breath diffusion of carbon monoxide did not reflect progression of melanoma but suggested that this effect was more likely to be explained by BCG pneumonitis [233].

2. Radionuclidic Impurities

The percent efficiency of the GeLi detector as measured by standard point sources appears in Table 8.

The 0.5 μCi sample of decayed Ga-67-citrate counted for four minutes showed only seven peaks with less than a 10% error. The first six listed in Table 9 could be attributed to Ga-67 while the seventh peak was K-40.

It appears that there were no significant long lived radionuclidic impurities in the preparation, albeit a short time was used to measure this. Preliminary 10 hour studies on a NaI crystal indicated that there were peaks when background was subtracted from the sample, but the resolution was considered too poor for identification. These data would indicate that the commercially available Ga-67-citrate (C.E. Frosst) sample was apparently free from long-lived radionuclidic impurities.

3. Autoradiography

The previous data on biodistribution of Ga-67-citrate and its correlation with histopathology indicated that BCG treatment alters the biodistribution of radiogallium by the development of systemic granulomatosis. In Figure 10 an H-E stained liver section shows three distinct areas associated with a BCG induced granuloma. The central portion of the lesion was filled with an accumulation of macrophages and necrotic tissues. Using Ziehl-Neelsen staining, acid-fast bacilli could be readily identified in this area. The

Table 8. Photon Energy and Corresponding Efficiency of Detection in a WIN-15 GeLi Detector

Energy (KeV)	Efficiency (%)
88	0.13410
122	0.13318
392	0.03595
662	0.02437
898	0.01457
1173	0.01321
1332	0.01172

Table 9. Photon Energies Observed from a Sample of Ga-67 Decayed for 30 days

Energy (KeV)	Area (c/4 min) A		% Error ($\frac{\sigma}{A}$ x 100)
92.72	26,000	$\sigma = A + 2B$	0.8
94.74	249,285	B = background	0.2
186.04	101,356		0.3
210.42	8,120		1.4
301.68	47,143		0.5
394.96	11,284		1.0
1461.73	229		7.4

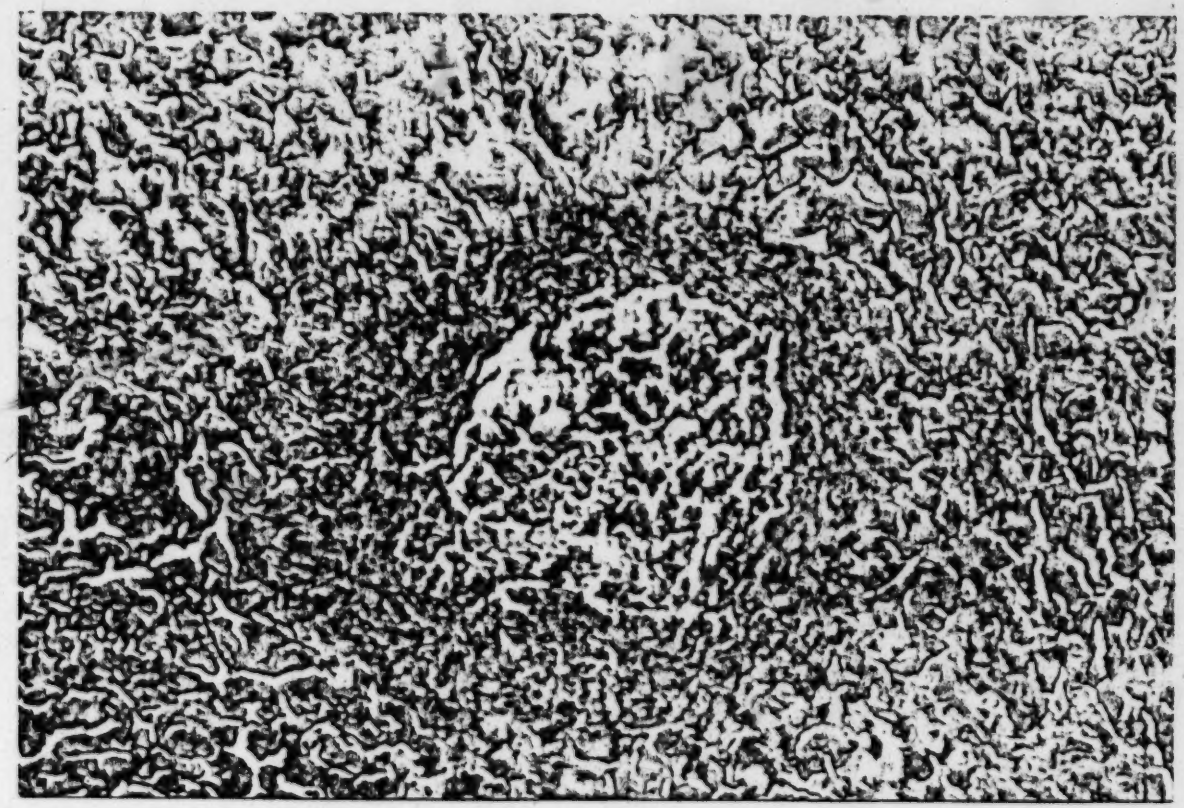


Figure 10. Liver granuloma from BCG treated mouse (H-E stain x 100). Three distinct areas are evident:

a) A central portion of the lesion filled with an accumulation of macrophages. Acid fast bacilli can be identified here.
b) Surrounding the central core is a heavily staining layer of fibrous cells with some infiltration of lymphocytes.
c) Normal hepatic tissue.

granulomatous lesion was bordered by a heavily staining layer of fibrous cells with infiltration by some lymphocytes. Autoradiographic examination (Figure 11) indicates an intense uptake of radiogallium by the interior of the lesion with very little or no accumulation of the radiotracer by the layer of fibrous cells. Numerous granulomata of this type were readily evident throughout the liver of the BCG treated mice. Histological sections of the spleen in BCG treated mice showed many diffuse lesions and necrotic areas which were not as well defined as those occurring in the liver, but with correspondingly large accumulations of Ga-67 autoradiographically visible. Because of the relatively low concentration of radiogallium by the kidneys and lungs, autoradiographic evidence was not as definitive in these tissues although lesions were seen histologically. No such abnormal foci of radioactivity were observed in control mice. The responses of the mice to intradermal BCG in this study were qualitatively similar to those observed by Ueda and co-workers in 1978[225] following intravenous administration of BCG in that granulomatous lesions were readily produced in lung, liver, spleen and kidneys. Similarly, the mouse animal model simulated the human response where hepatic[159, 230] and splenic[232] granuloma were reported following BCG immunotherapy. The autoradiographic study illustrates that the macrophage laden core of the BCG induced granulomatous lesion is particularly avid for radiogallium. This observation is consistent with the findings of Tsan and co-workers in 1978[96] who noted the high uptake of Ga-67 by non-viable polymorphonuclear leukocytes and with those of Ostroy, Johnston and Gams in 1979[88] who

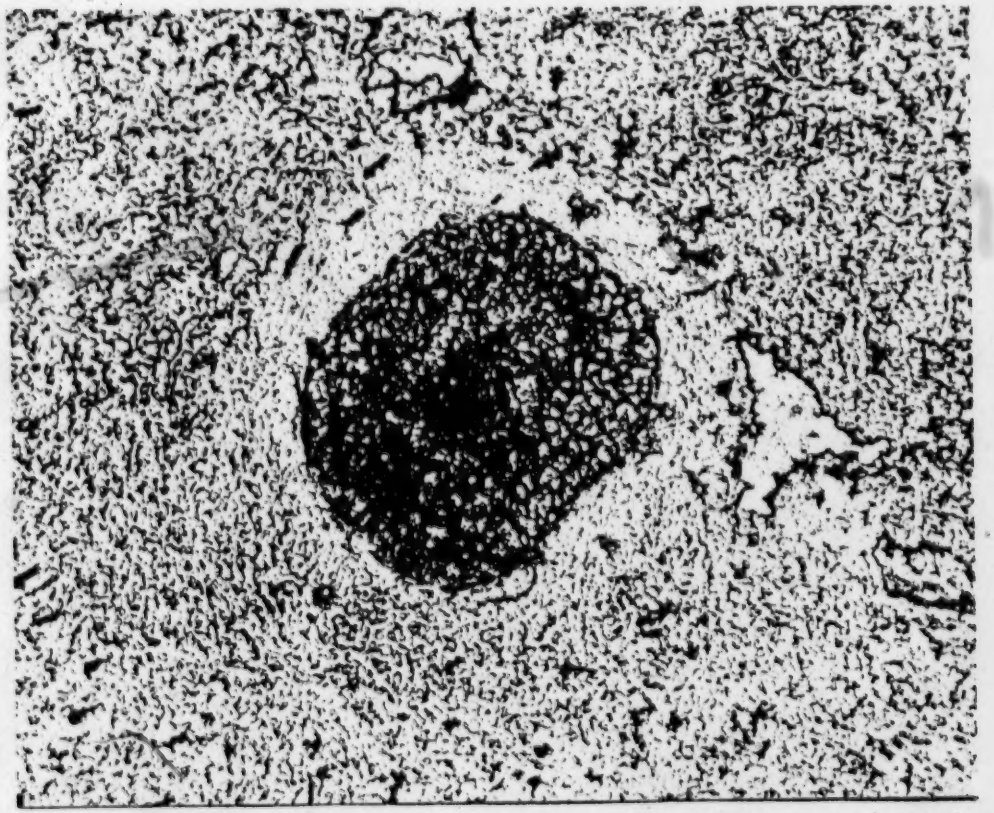


Figure 11. Autoradiography of a liver granuloma from BCG treated Mouse (x 40). An intense accumulation of radiogallium in the macrophage laden interior of the lesion. Virtually no uptake of Ga-67 is seen in the surrounding fibrous layer. A diffuse uptake is seen in the nondiseased hepatic tissue.

reported that gallium-67 incorporation into human granulocytes is greatly increased by hypoxic conditions such as would be found in inflammatory lesions and abscesses. Extrapolation of the results seen in this paper to a clinical setting where it is well established that Ga-67-citrate localizes in tumour tissue as well as at inflammatory sites, would indicate that caution should be used in the interpretation of radiogallium scans in cancer patients on BCG immunotherapy. Because the liver and spleen normally show a high accumulation of Ga-67, increased uptake in these organs may be difficult to assess during clinical Ga-67 scanning. However, the possibility of increased pulmonary uptake of ^{67}Ga due to BCG induced granuloma should be considered. Increased diffuse pulmonary uptake on Ga-67 scans has been reported in the presence of tumours, inflammatory reactions and infections including BCG induced pneumonitis.

4. Biokinetics of Whole Body Elimination

Whole body counting would seem to indicate that BCG does not alter the overall rate of elimination of radiogallium from mice. The average whole body retention for each day plus or minus the standard deviation is presented in Table 10. This table illustrates the percent of the injected activity for 8 control and 12 BCG treated mice two weeks after the BCG treatment regimen. The mice were injected with 2 μCi of gallium-67-citrate and individually assayed in the whole body counter described previously.

Table 10. Whole body retention of ^{67}Ga-citrate in control and BCG treated mice *

Days after injection of Ga-67-citrate	Control Mice (n=8)	BCG Treated Mice (n=12)
0	100	100
1	54.3 ± 4.6	45.2 ± 10.6
2	46.0 ± 3.7	38.9 ± 9.2
3	37.9 ± 6.0	33.4 ± 11.5
4	35.7 ± 2.8	30.2 ± 8.6
5	-	-
6	-	-
7	33.9 ± 3.4	27.5 ± 7.7
8	31.0 ± 4.3	26.4 ± 7.6
9	30.5 ± 4.0	25.9 ± 6.7
10	26.4 ± 1.7	24.2 ± 6.8
11	28.8 ± 3.7	23.3 ± 8.5

* Expressed as mean % ± S.D. of initial whole body radioactivity.

2 µCi injected i.v. two weeks after BCG treatment regimen.

As can be seen, the mean ± S.D. overlap in all cases indicating that there was no statistical difference in the overall rates of elimination of gallium from the BCG and control treated animals. The pooled data presented in Table 10 seemed to fit a model with two elimination rates when curve stripping was performed. An algorithm for least squares estimation of nonlinear parameters[235] was employed to arrive at the first estimate parameters.

The initial estimates as determined from a least squares estimation of nonlinear parameters was employed along with the pooled data of Table 10 to arrive at the final estimates of first and second component magnitudes and biological half-lives presented in Table 11 by nonlinear regression analysis[236]. The advantage of using pooled data for fitting to a nonlinear equation is that of less scatter as the fitting process cannot tell the difference between one or more mice. During the first day approximately half of the injected activity was excreted. As no other measurements were made during this first day an initial fast component may not have been detected.

The data obtained from the control and BCG treated mice were very nearly the same. The model used would be a single compartment with two elimination rates. It would be incorrect to ascribe physiological parameters to these two components as, owing to the small size of the mouse, urine and feces were not collected.

5. <u>In Vitro Uptake of ^{67}Ga by Membrane Filter</u>

This study was done to determine to what extent the filters themselves took up radiogallium. The statistical analysis used

Table 11. Final Estimates of First and Second Component Magnitudes and Biological Half-Lives of Intravenously Injected Ga-67-citrate

	Control Mice	BCG Mice
Component 1		
Magnitude	56.7%	62.2%
Half Life	0.500 day	0.376 day
Component 2		
Magnitude	43.0%	37.7%
Half Life	17.1 days	15.8 days

indicated a measurable difference in uptake ($p < 0.05$) for different rinse volumes and for different combinations of the triple interaction of volume, activity and rinse ($p < 0.01$). The larger the volume of rinse used, the smaller the activity retained on the filter. Using Duncan's New Multiple Range Test a rinse volume of 1.6 ml, which gave an averaged uptake of 4.01%, was found unacceptable and to differ significantly from rinse volumes of 5 ml or 10 ml which gave averaged uptakes of 1.91% and 1.67% respectively. The combination of 0.1 ml filtered, 0.25 µCi of Ga-67 and 10 ml of rinse gave the lowest averaged uptake by the filter membrane, being 1.07% while 1.0 ml filtered, 2.0 µCi absolute activity, and 1.6 ml rinse had a percent uptake of 9.24%. There was no significant trend observed for the other sources of variation. It was decided, for further experiments, to use a combination of 2 ml of incubation media filtered, a maximum counting activity and a rinse volume of 10 ml, which gave an averaged estimated uptake of 1.12% in the membrane filter.

6. In Vitro Uptake of Ga-67-Citrate by BCG

Figure 12 illustrates the *in vitro* incorporation of Ga-67 by BCG over a 4 hour time period. The live suspension of BCG showed rapid uptake in the first 15 minutes of incubation and reached 15.2 ± 3.7% of the total Ga-67 radioactivity in the incubation media at 3 hours. Under the conditions of this experiment, i.e. using a concentration of 2 µCi/ml in Hank's solution, filtering a 2 ml aliquot followed by a wash with 10 ml of Hank's solution the uptake of Ga-67 by the membrane filter in the absence of any bacteria was 2.41 ± 1.38%

(mean ± S.D. of 10 determinations) of the total radioactivity filtered. Preliminary experiments indicated that the % uptake by the membrane filter was not significantly altered by changes in the Ga-67 concentration of the filtered solution within the range of 0.1 to 2.0 μCi/ml or by the radioactive solution filtered within the range of 0.1 to 2.0 ml. Presoaking the membrane filters in non-radioactive $GaCl_3$ (1.5 mM) produced no significant alteration in the amount of radioactivity retained on the filter. After filtering 2 ml of the BCG suspension containing 10^8 cells/ml the BCG organism could be readily identified on the membrane by fluorescent microscopy. This was to be expected as the organism is 0.5 μm in diameter by 3 μm long. The BCG organisms were effectively held on the filters as demonstrated by fluorescent microscopy of the face and reverse side of the filters and by colony counting on Mark's modification of Lowenstein-Jensen media with pyruvic acid. The filtrate was sterile of anerobic and aerobic organisms as determined by sterility counts on blood sugar plates and McKonkey plates. In contrast to the live BCG suspension, the heat killed BCG did not show any significant uptake of Ga-67-citrate from the incubation media at any time interval.

The binding of Ga-67 to the membrane filter seen in these experiments was also reported by Driedger[236] and by Tsan and co-workers[96]. However, under controlled laboratory conditions the amount of this non-specific binding was relatively constant and reproducible. Using the incubation procedures of Tsan[96] and colleagues but employing the membrane filtration technique instead of their centrifugation steps for separating the cells and the

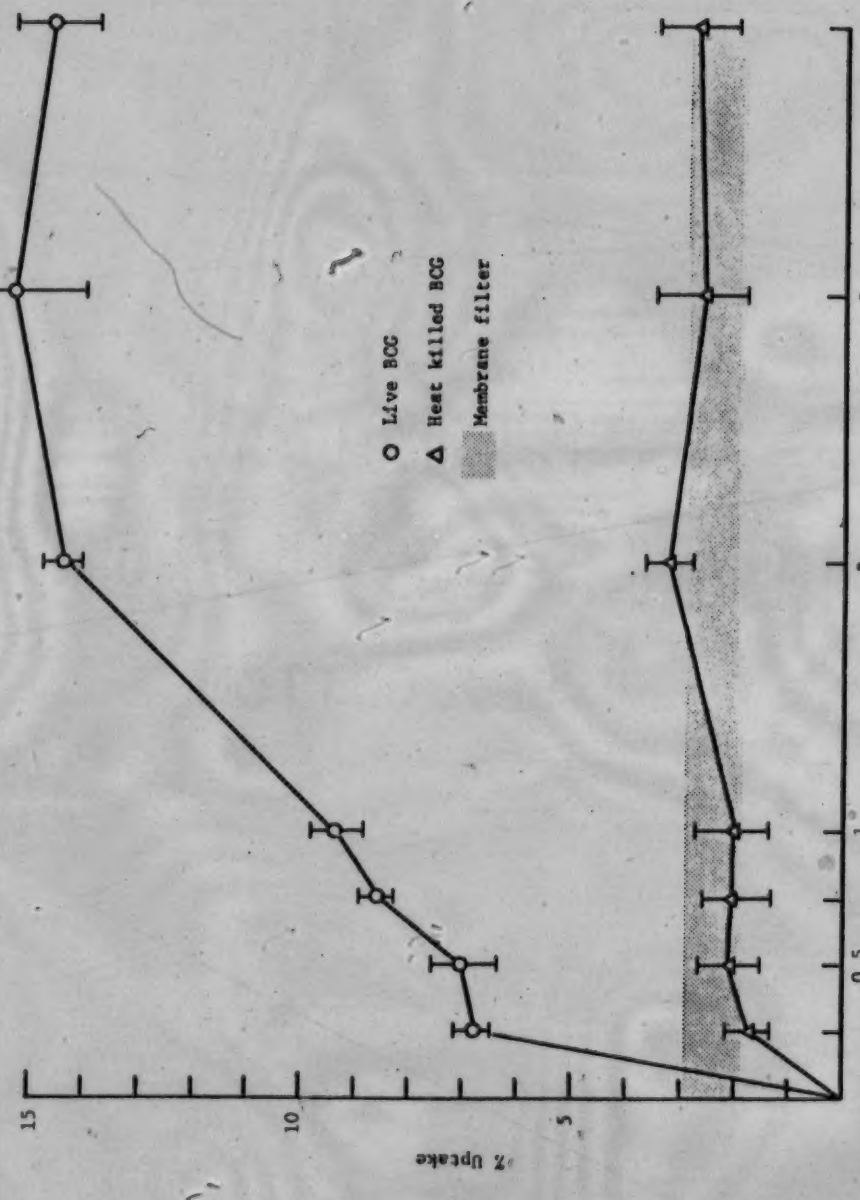


Figure 12. In vitro uptake of Ga-67 citrate by BCG; each point represents the mean ± S.D. of 4 determinations.

Ga-67 incubation media, we were readily able to duplicate their experiments on the uptake of Ga-67 by Staphylococcus aureus.

On the basis that bacteria are a common component of inflammatory lesions, Menon, Wagner and Tsan investigated the uptake of Ga-67 by several microorganisms and found significant uptake by Staphylococcus aureus[234]. The observation that large numbers of acid-fast bacilli were present in the BCG induced granuloma in mice prompted the *in vitro* uptake studies which provided data that viable BCG cells can significantly incorporate radiogallium. Similar to the findings, of Menon and co-workers with Staphylococcus aureus[224], the uptake of Ga-67 by heat killed BCG was minimal. Although there is no direct evidence that viable BCG cells in the inflammatory lesion contribute significantly to accumulation of radiogallium at that site, further research into the area of *in vivo* uptake of Ga-67 by microorganisms seems warranted.

V. CONCLUSIONS

The data obtained from the experimental portion of this thesis indicate that:

1. BCG immunotherapy in mice may significantly alter the biodistribution of radiogallium.

2. The effects of BCG on the biodistribution of radiogallium in mice are evident even at 2 days following BCG therapy but appear more obvious 4 weeks after the BCG therapy regimen.

3. There is a good correlation between the increased uptake of radiogallium and incidence of histopathological abnormalities in each of the same tissues sampled.

4. Autoradiography confirmed that the altered biodistribution was chiefly due to the development of granulomata which were avid for radiogallium.

5. Whole body counting techniques did not show any overall difference in the rate of elimination of radiogallium from BCG treated and control mice two weeks after the BCG therapy regimen.

6. In vitro membrane filters have been shown to bind gallium nonspecifically to a limited extent.

7. Viable BCG organisms accumulate radiogallium in in vitro incubation, but heat killed BCG show no avidity for Ga-67.

8. No significant amounts of long lived radionuclidic impurities could be found in the commercially available Ga-67-citrate.

9. These findings indicate the need for caution in interpreting radiogallium scintiscans in patients receiving BCG immunotherapy.

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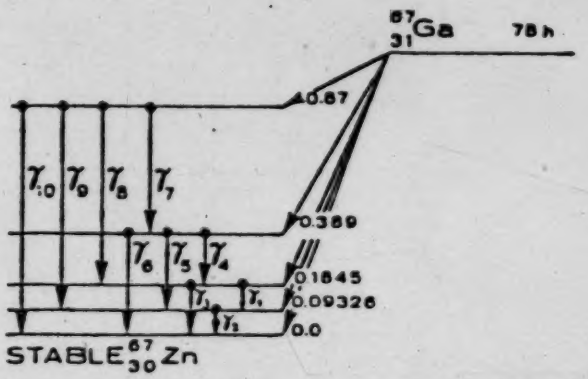
Appendix I: Decay Scheme and Nuclear Data

GALLIUM-67

ELECTRON CAPTURE DECAY



OUTPUT DATA

Radiation (i)	Mean number/ disintegration (n_i)	Mean energy (MeV) ($\bar{E}_i$)	Δ_i $\left(\frac{\text{g-rad}}{\mu\text{Ci-h}}\right)$
Electron capture	—	—	*
Gamma-1	0.0225	0.0912	0.0044
K int. con. electrons	0.0017	0.0815	0.0003
L int. con. electrons	0.00015	0.0901	0.0000
Gamma-2	0.3962	0.0933	0.0789
K int. con. electrons	0.2496	0.0836	0.0445
L int. con. electrons	0.0267	0.0922	0.0053
M int. con. electrons	0.0089	0.0932	0.0018
Gamma-3	0.2294	0.1845	0.0903
K int. con. electrons	0.0025	0.1748	0.0009
L int. con. electrons	0.00015	0.1834	0.0001
Gamma-4	0.0150	0.2060	0.0066
K int. con. electrons	0.0004	0.1963	0.0002
Gamma-5	0.2053	0.2960	0.1297
K int. con. electrons	0.0006	0.2863	0.0004
Gamma-6	0.0800	0.3880	0.0662
K int. con. electrons	0.00015	0.3783	0.0001
Gamma-7	0.0022	0.4900	0.0023
Gamma-8	0.0010	0.6900	0.0015
Gamma-9	0.0010	0.7800	0.0017
Gamma-10	0.0019	0.8700	0.0035
X-rays: $K\alpha$-1	0.2912	0.0086	0.0053
$K\alpha$-2	0.1453	0.0086	0.0027
K3-1	0.0524	0.0096	0.0011
K3-2	0.0009	0.0097	0.0000
Auger electrons: KLL	0.5136	0.0075	0.0082
KLX	0.1397	0.0085	0.0025
KXY	0.0067	0.0095	0.0001
LMM	1.73	0.0009	0.0033
MXY	3.68	0.0001	0.0008

* For reference only—does not contribute to dose.

INPUT DATA

Radiation	%/disintegration	Transition energy (MeV)	Other nuclear parameters
Electron capture-1	0.6	0.13	Allowed
Electron capture-2	30.	0.611	Allowed
Electron capture-3	24.	0.8155	Allowed
Electron capture-4	45.	0.9067	Allowed
Electron capture-5	0.4	1.0	Allowed
Gamma-1	2.44	0.0912	M1, $\alpha_K = 0.074$
Gamma-2	68.14	0.0933	E2, $\alpha_K = 0.63$, $K/(L+M) = 7.0$
Gamma-3	23.21	0.1845	M1 + 28% E2, $\alpha_K = 0.011$, $K/(L+M) = 13.0$
Gamma-4	1.55	0.206	M1, $\alpha_K = 0.029$, $K/(L+M) = 14.0$
Gamma-5	20.6	0.296	M1, $\alpha_K = 0.0029$, $K/(L+M) = 8.0$
Gamma-6	8.02	0.388	M1, $\alpha_K = 0.0019$
Gamma-7	0.22	0.49	M1, $\alpha_K =$ 0.00185 (T), $\alpha_L =$ 0.000087 (T)
Gamma-8	0.1	0.69	M1, $\alpha_K =$ 0.000482 (T), $\alpha_L =$ 0.0000409 (T)
Gamma-9	0.1	0.78	M1, $\alpha_K =$ 0.000369 (T), $\alpha_L =$ 0.0000315 (T)
Gamma-10	0.19	0.87	M1, $\alpha_K =$ 0.000293 (T), $\alpha_L =$ 0.0000251 (T)

Ref.: Lederer, C. M. et al, Table of Isotopes, 6th ed.
(T) = Theoretical value.